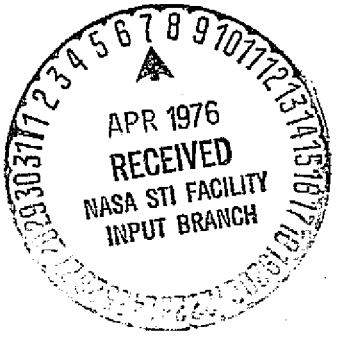
Final Report

ELECTROPHORETIC SEPARATION OF PROTEINS IN SPACE

NASA Contract No. NAS 8-29823



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Advice for the Reader

The various sections of this report are apt to be of differing degrees of interest for a given reader. Please use the Table of Contents. Many of the longer sections are preceded by a Summary which may help the busy reader determine if he should delve into the details.

The section on ampholyte analysis is abstracted from a paper which has been accepted for publication in Analytical Biochemistry.

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*While this work was not supported by NASA funds, it is a natural outgrowth of the project and indicates one direction of future interest.

I. AMPHOLYTES

A. ANALYSIS

Summary

Commercially available and synthetic wide range and short range ampholytes used in the isoelectric focusing of proteins can be analyzed by ion exchange chromatography. A pH gradient over the pH range 3.8 to 11.0 was used to elute the ampholytes from a column of a sulfonated polystyrene resin. The wide range ampholytes were resolved into some 60 to 70 ninhydrin positive components. The recovery obtained with the method was quantitative. Acid short range ampholytes have approximately 35 components which elute readily from the ion exchange resin. Basic short range ampholytes gave about 50 components, most of which eluted at alkaline pH.

Background

Isoelectric focusing in stabilized pH gradients is one of the most powerful techniques for the isolation and characterization of biological macromolecules. The artificial pH gradients can be formed by mixtures of synthetic ampholytes possessing closely-spaced isoelectric points covering the required range of pH (Catsimpoolis, 1973). The commercially available carrier ampholytes are complex mixturesof low molecular weight polyamine polycarboxylic acids (Vesterberg, 1973). It is evident that the formation of reproducible pH gradients is of paramount importance in the successful focusing of proteins and other macromolecules and that it is related to the reproducibility in the synthesis of ampholytes. Control of the latter requires a reliable method for the quantittative analysis of the ampholyte mixtures. The commercially available ampholytes are known to be complex mixtures from dye staining patterns (Frater, 1970; Radola, 1973), refractive index variations (Righetti et al., 1975), and caramelization patterns (Felgenhauer and Pak, 1973).

Method

The method for the quantitative analysis of synthetic ampholytes developed under the contract uses ion-exchange chromatography on a column of a sulfonated polystyrene resin with a pH and ionic strength gradient. The following buffers were used to prepare the elution gradient. (A) pH 3.80 citrate buffer containing 14.71 g Na_3citrate·2 H_2O, 25.0 ml of 2.00 N NaOH, 5 ml thiodiglycol, titrated to pH 3.80 with 6 N HCl and diluted to 1 liter. (B) pH 5.00 citrate buffer containing 14.71 g Na_3citrate·2 H_2O, 25.0 ml of 2.00 N NaOH, 35.07 g NaCl, titrated to pH 5.00 with 6 N HCl and diluted to 1 liter. (C) pH 7.50 phosphate buffer prepared by mixing a 0.05 M Na_2HPO_4 solution 0.75 M in NaCl with a 0.05 M Na_2HPO_4 solution 0.70 M in NaCl until a pH of 7.50 is reached. (D) pH 9.4 borate buffer prepared by titration of a solution 0.05 M in H_3BO_3 and 0.8 M in NaCl with a 0.2 M NaOH solution 0.6 M in NaCl to pH 9.4. (E) Alkaline phosphate buffer prepared by mixing 900 ml of a solution 0.05 M in Na_3PO_4 and 0.65 M in NaCl with

100 ml of a solution 0.05 M in Na_2HPO_4 and 0.70 M in NaCl. (F) 0.2 N NaOH. To each liter of buffer were added 10 ml of Brij 35 solution (100 gm plus 200 ml H_2O) and to each buffer except (F) 100 µl of octanoic acid.

A 9-chamber gradient maker was filled as follows: Chamber 1 - 55 ml pH 3.8 buffer and 20 ml pH 5.0 buffer; Chamber 2 - 15 ml pH 3.8 buffer and 60 ml pH 5.0 buffer; Chamber 3 - 75 ml of pH 5.0 buffer; Chamber 4 - 20 ml pH 5.0 buffer and 55 ml pH 7.5 buffer; Chamber 5 - 75 ml of pH 7.5 buffer; Chamber 6 - 35 ml pH 7.5 buffer and 40 ml pH 9.4 buffer; Chamber 7 - 75 ml of pH 9.4 buffer; Chamber 8 - 75 ml of alkaline phosphate buffer; Chamber 9 - 75 ml of 0.2 N NaOH.

Approximately 6 mg of the ampholyte mixture (calculated as dry weight) in 500 µl of pH 3.8 citrate buffer was placed on a 0.5 cm x 135 cm column of sulfonated polystyrene ion exchange resin (Aminex A-6, Bio-Rad) maintained at 60° C and previously equilibrated with the pH 3.80 buffer. The buffers from the gradient maker were pumped through the system at a pressure of 180-200 psi and flow rates of about 30 ml/hr. The effluent was sampled continuously, mixed with 2.88 volumes of ninhydrin reagent (40 g ninhydrin, 3 g hydrindantin, 5.3 l methyl cellosolve, 4 l water, 700 ml of 4 M sodium acetate buffer, pH 5.5) and the absorbance measured at 550 nm. A Technicon amino acid analyzer was used for the study. A stream-splitting arrangement was used to collect a portion of the column effluent in 3 ml fractions. The pH of the fractions was measured with a glass electrode and a Radiometer PHM4 pH meter.

Analysis of Commercial Ampholytes

Three lots of commercial wide range ampholytes (LKB lots #16, 17 and 52) were analyzed by the foregoing method. The results of the analyses of LKB ampholines lots #17 and 52 are shown in Fig. 1 A and B, respectively. Lot 16 was almost identical with lot 17. Approximately 62 peaks are seen in both mixtures over the pH range 4 to 11. Most of the components are eluted over the pH range 4 to 7. The results of analysis of LKB pH 8 to 9.5 (lot #3) ampholine and LKB pH 4 to 6 ampholine (lot #7) are shown in Fig. 2 A and B, respectively. The acidic ampholyte consisted of about 35 components while the basic ampholyte consisted of about 52 components. Most of the acid short range ampholyte components are eluted in the first 230 ml while only a very small proportion of the components of the basic ampholytes are eluted in this volume. The broad peak at about 470 ml observed in most of the chromatograms, but which is most prominent in the chromatograms of the short range ampholytes (Fig. 2) is a baseline artifact due probably to the elution of ammonia. The reproducibility of the elution volumes of the most prominent peaks in the chromatograms was $\pm$ 3 ml.

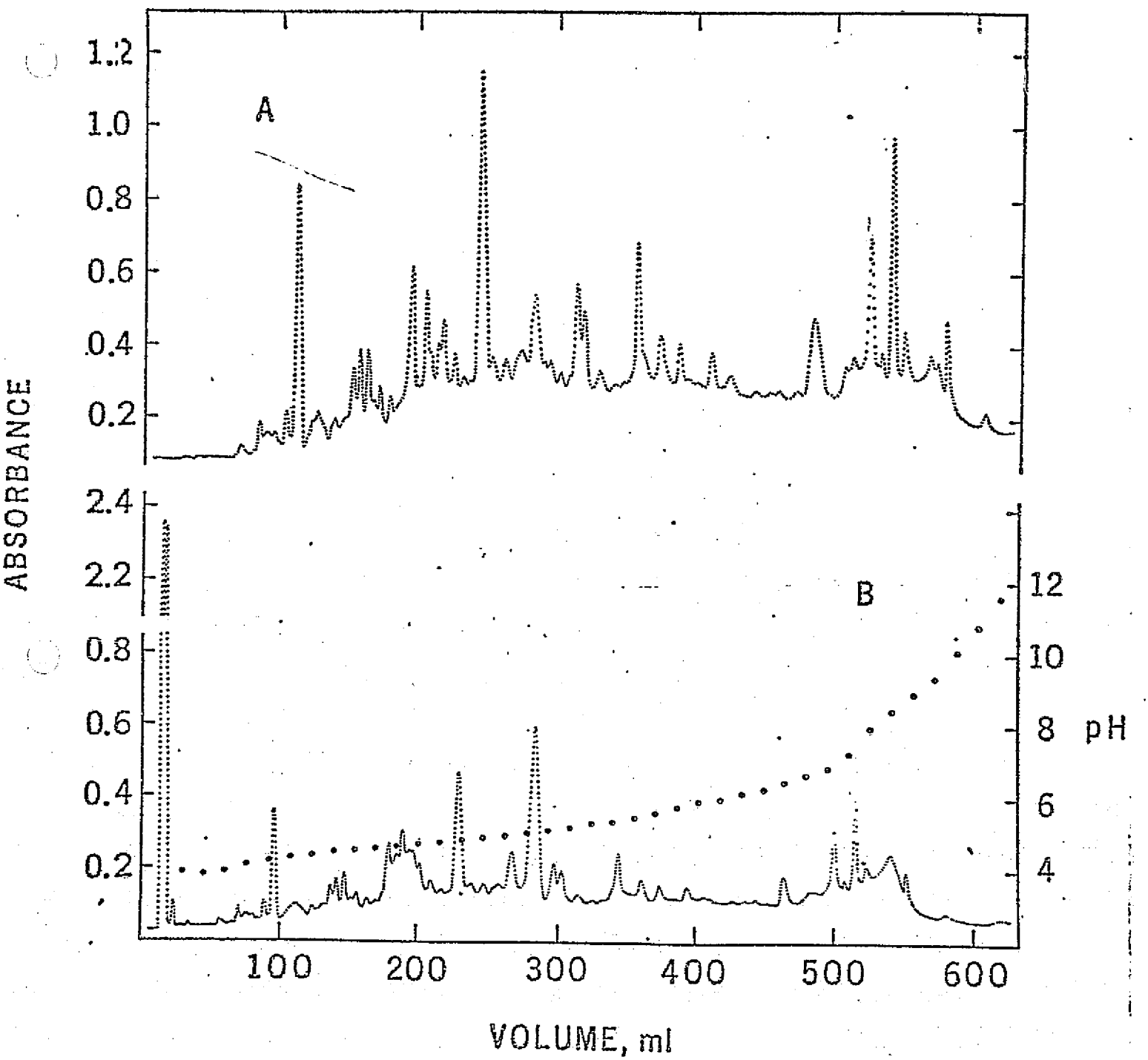


Figure 1. Elution patterns of ninhydrin positive components of wide range (pH 3-10) LKB ampholines, #17 (A) and #52 (B). The pH gradient employed is shown by the filled circles.

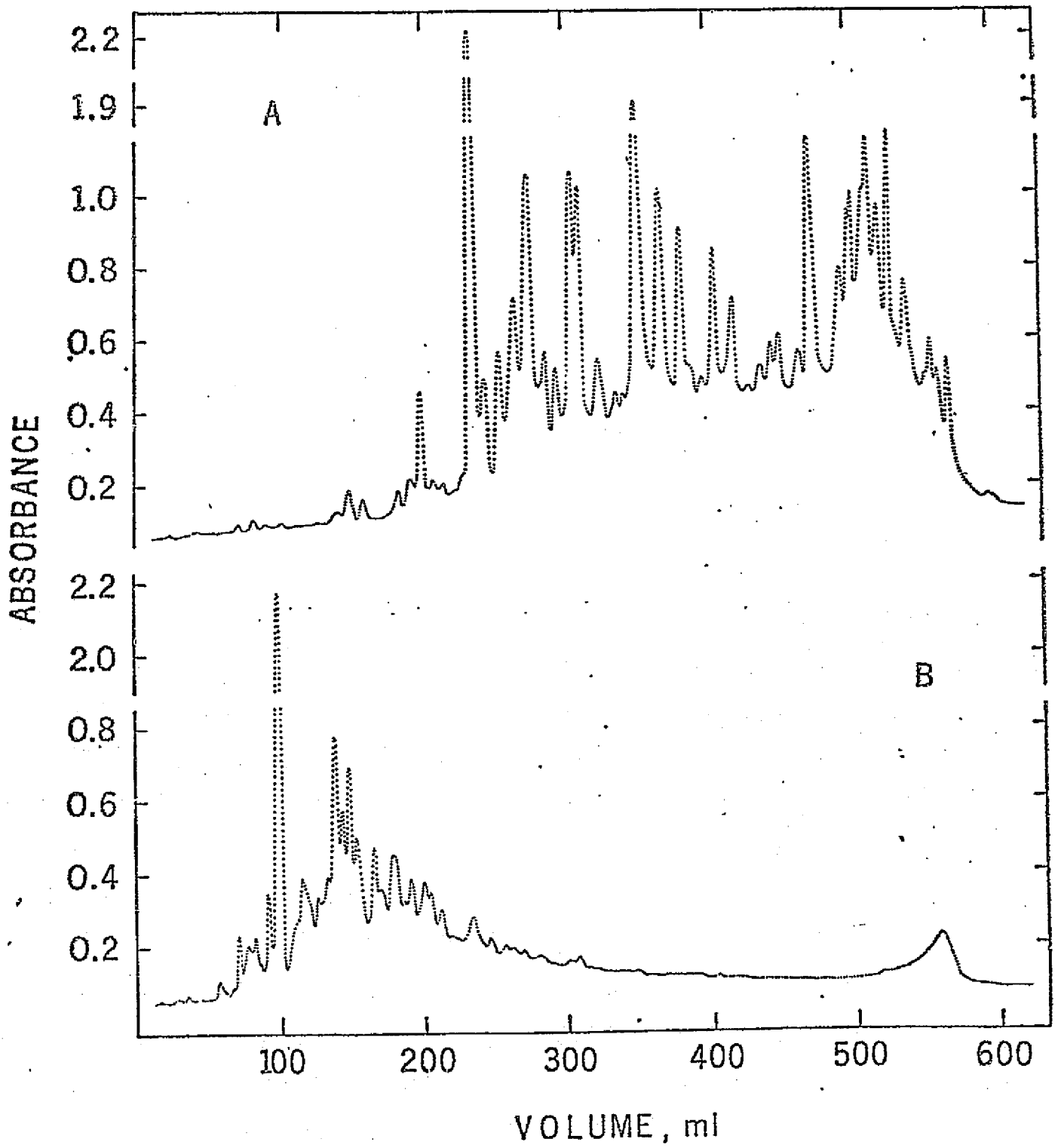


Figure 2. Elution patterns of ninhydrin positive components of short range LKB ampholines, #3 (pH 8-9.5) (A) and #7 (pH 4-6) (B).

The ninhydrin color yields of the ampholytes were measured in the various buffers used to form the gradient. They varied ± 5% from the average. The color yield was slightly lower in the alkaline buffers. The recoveries obtained with commercial ampholytes as well as with ampholytes synthesized by us were usually quite good. In a second type of recovery experiment, the gain in weight of the resin after the application of a large excess of ampholyte was found to be only 0.2 mg. This result is within the error of weighing and would represent 0.04% of the added ampholyte; hence, no ampholyte appeared to be irreversibly bound to the ion-exchange resin.

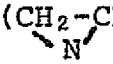
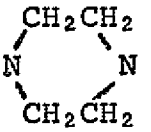
B. SYNTHESIS

Summary

Ampholytes were prepared from two simple triethylene tetramines and found to consist of only a few major components. Used in gels they give non-linear pH gradients which may be useful for preparative isoelectric focusing. They provide material for fractionation studies designed to prepare individual ampholyte species as spacers and for isotachyphoresis.

Ampholytes were also prepared from pentaethylene hexaamine, a complex mixture of isomeric amines. Charcoal treatment to remove substances absorbing at 280 nm was developed. The pH gradient generated by the ampholytes depends on the ratio of amine to acrylic acid used in synthesis. Short range basic and acidic ampholytes can be generated by using appropriate ratios.

Background

In order to obtain the large amounts of ampholytes required for preparative work and to understand the chemistry and properties of specific ampholyte components, a program in syn thesis was undertaken. Early steps had previously been taken by other members of this department (Vinogradov, 1973) following the method of Vesterberg (1969). The ampholytes were prepared by the reaction of acrylic acid (CH_2=CH COOH) with oligopolyethylene polyamines (NH_2-(CH_2-CH_2-NH)$_n$H). The latter are prepared commercially by polymerization of ethylene imine (). A wide variety of compounds are formed including linear and branched polymers and those containing piperazine rings  (Spell, 1969). The polyamines are partially purified by fractional distillation to yield the commercial products diethylene triamine (DETA), triethylene tetraamine (TETA), tetraethylene pentamine (TEPA), pentaethylene hexamine (PEHA) and hexaethylene heptamine (HEHA). Each fraction is a mixture. DETA has 3 components, TETA 7, TEPA 12

and PEHA 20 (Bergstedt and Widmark, 1970). Individual components can be partially separed by fractional precipitation with organic solvents (Glerup et al., 1970), cation exchange chromatography (Parrish, 1965) or preparative gas-liquid chromatography (Spell, 1969).

Preparation of Ampholytes from Triethylene Tetramine (TETA).

Ampholytes were prepared from triethylene tetramine and from two of its components 1, 4, 7, 10 tetraazodecane (TRIEN), $NH_2(CH_2)_2$-$NH(CH_2)_2NH(CH_2)_2NH_2$ and tris (2-aminoethyl) amine, (TREN), $(CH_2CH_2NH_2)_3N$. The latter were separated by fractional precipitation of the chloride in water-ethanol mixtures. In a typical preparation of TETA ampholyte 55 ml of vacuum distilled (97-110° at 360 microns) TETA was diluted with 105 ml water and 55 ml of acrylic acid was added over a 50-min period under nitrogen. The mixture was heated at 70° overnight. Completion of reaction was assayed by determining residual acrylic acid by titration with permanganate.

Characterization of TETA Ampholytes

The materials were assayed by ion exchange chromatography. TETA ampholyte (Fig. 3) contains 13 major components. TREN ampholyte has 4 components. These correspond with peaks (indicated by ↑ in Fig. 3) in the parent mixture. TRIEN ampholyte gave 7 to 9 peaks (↑). The small number of components makes these ampholytes especially useful for studies of their effect on pH gradients, their distribution in isoelectric focusing and their fractionation.

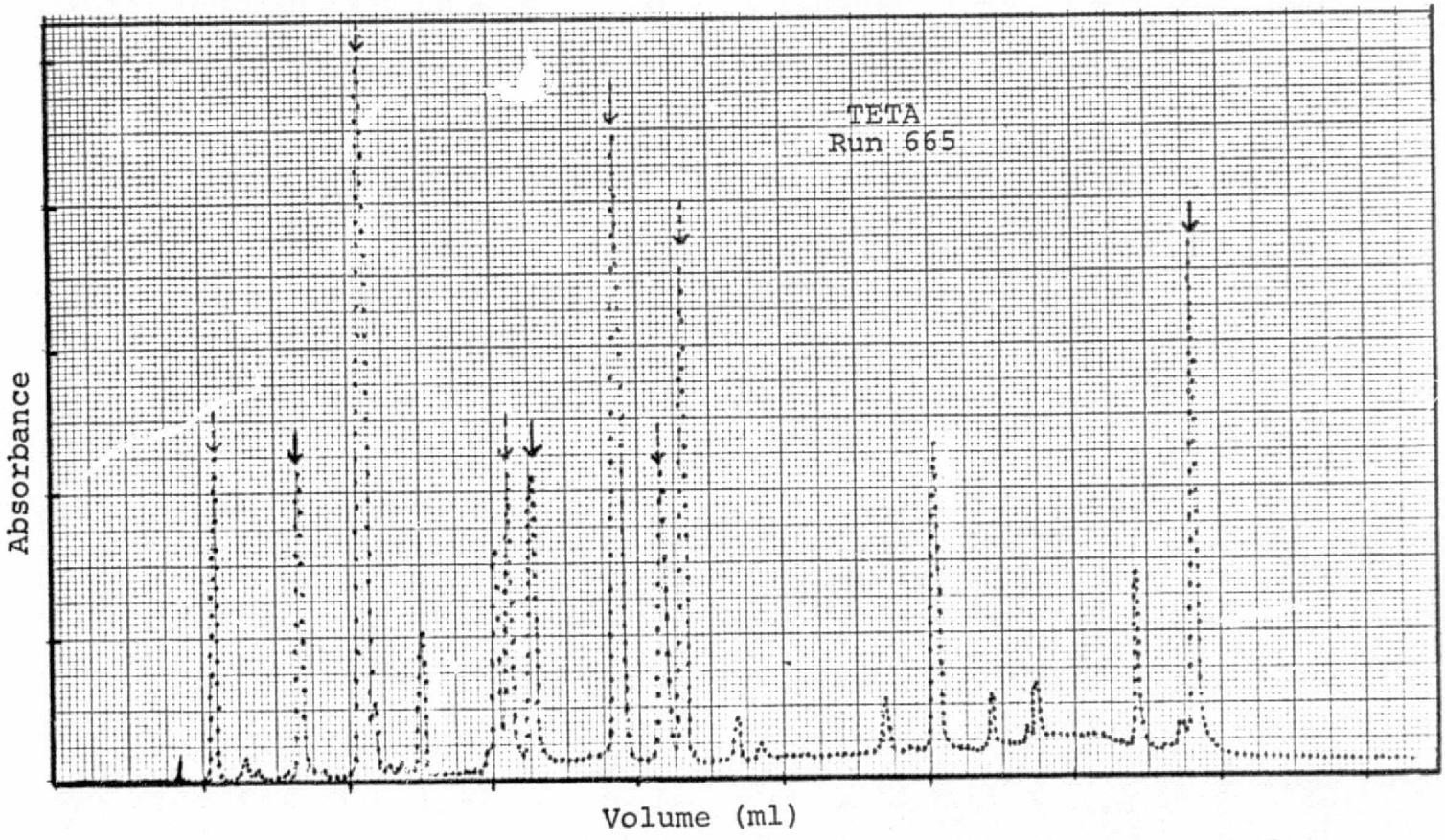


Fig. 3. Analysis of TETA ampholyte peaks found also in TREN ampholyte are indicated by (↑) and those in TRIEN ampholyte by (↑).

Polyacrylamide gels were made with the ampholytes and the pH gradient along the gel determined (Fig. 4). The simpler mixtures give plateaus probably corresponding to the pI of major components. The TETA ampholyte gives a fairly linear gradient although small deviations occur corresponding to those found in the simpler mixtures (Fig. 4). While linear pH gradients are best for analytical isoelectric focusing, the non-linear ones are very useful for preparative work.

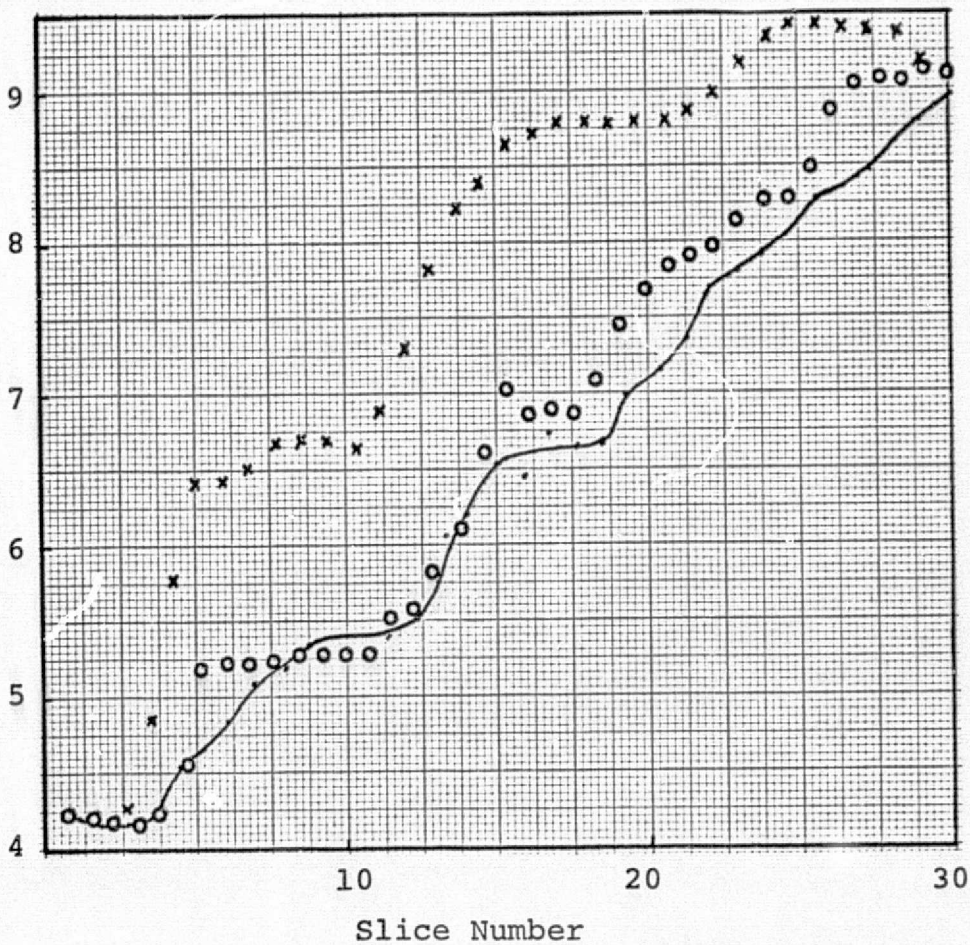


Fig. 4. pH gradients generated using simple ampholytes prepared from triethylene tetraamine (solid line) or from either of its major components TRIEN (O) or TREN (X).

Preparation of Ampholytes from Pentaethylene Hexamine (PEHA)

Considerable difficulty was encountered in preparing ampholytes from PEHA. Preparations tended to be yellow colored and to have an absorbance at 280 nm sufficiently great to interfere with protein analysis. Contrary to the experience of others precipitation as the hydrochloride (Vinogradov et al., 1973) and vacuum distillation even at very low pressures or under nitrogen (Righetti et al., 1975) were not useful although the latter yielded a series of colorless and yellow fractions. Precipitation by reacting with CO_2, by forming the hydrates at 4°, or by forming the tosyl derivative, was likewise ineffective. Charcoal treatment of the PEHA markedly reduced the 280 readings with minimal loss of material (recoveries averaged 100.8% by weight). It was convenient to treat the PEHA by running an aqueous solution slowly through a 2.5 x 21.5 cm column of 50-200 mesh cocoanut charcoal (Fisher 5-690) which had been previously washed with 1 N HCl and then with methanol. It was important to make the PEHA solution dilute. A 10% solution was quite satisfactory; 33%, 50% and undiluted materials retained appreciable impurity.

The process was little affected by temperatures from 0° to 37° and was usually done at room temperature. A 1% solution of PEHA had an absorbancy of .336 to .372 at 280 nm. This was routinely reduced to .08 and the ampholytes prepared had absorbancies as low as 0.033 which is half that of commerical ampholytes. The ampholytes were prepared as described for TETA ampholytes. Charcoal treatment of ampholyte solutions removes further material which absorbs at 280 nm without affecting the quality of the ampholyte.

Analysis of PEHA Ampholytes

The proportion of polyamine to acrylic acid was quite critical. Excess amounts of acrylic acid gave products which were narrow range acidic ampholytes while too little acrylic acid gave narrow range basic material (Fig. 5). The narrow range materials still contained a large number of components so that they should be good for separation work. It was necessary to titrate the purified PEHA to accurately determine its content of amino groups before calculating the amount of acrylic acid to add. The most satisfactory ampholytes have a ratio of 1.84 amino groups to one carboxyl group.

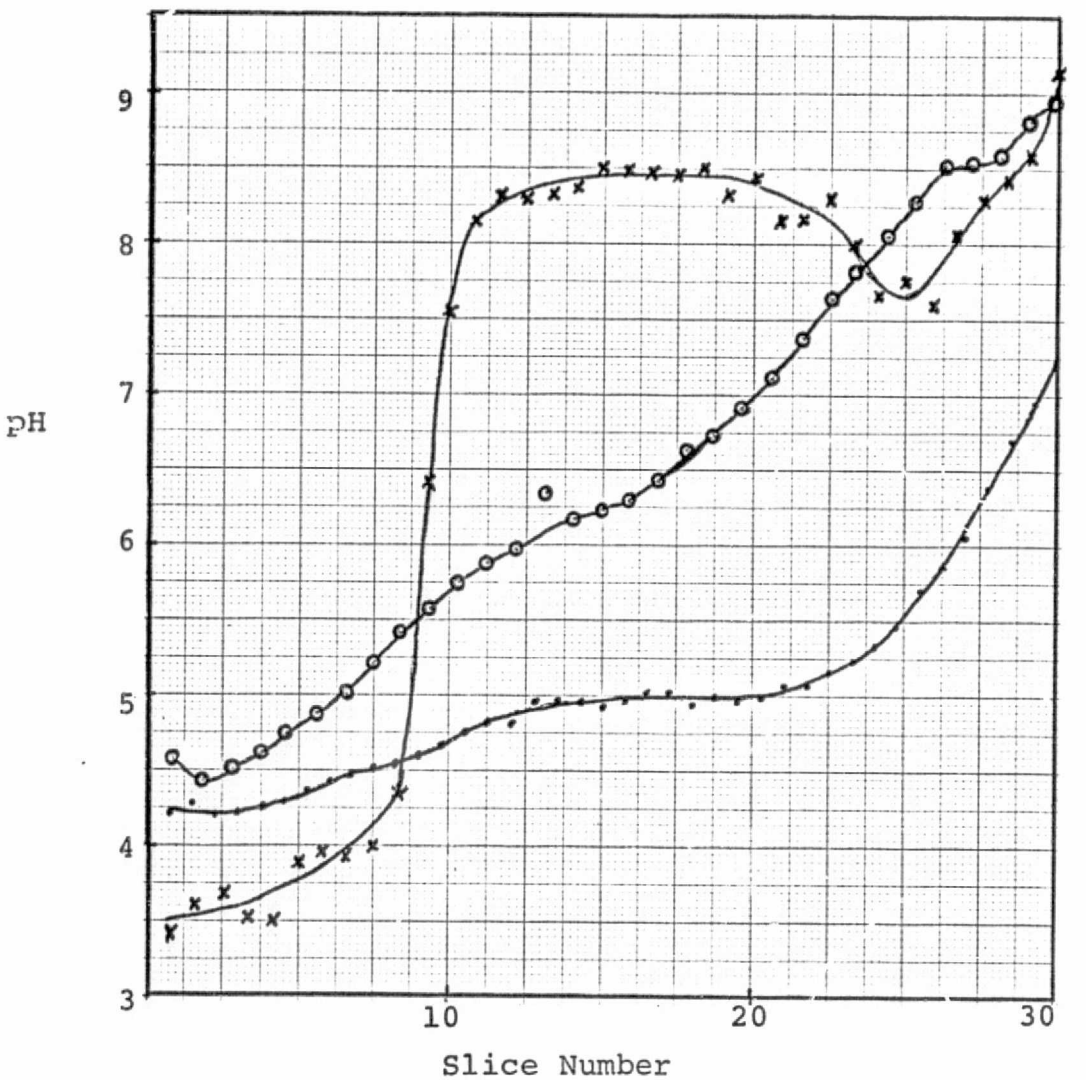


Fig. 5. pH gradients obtained with PEHA ampholytes prepared with different molar ratios of amino groups to acrylic acid.

X = 3.01 ⊙ = 1.84 • = .27

C. AMPHOLYTE DISTRIBUTION IN ISOELECTRIC FOCUSING

Summary

The spatial distributions of individual components of carrier ampholyte mixtures obtained by isoelectric focusing in cylindrical polyacrylamide gels and in a thin layer Sephadex gel were determined by ion exchange chromatography of the eluates of gel slices. A 1 mm slice of PAG separating the A and B forms of β-lactoglobulin (IP = 5.21 and 5.34, respectively) was found to contain at least 12-14 ampholyte components out of a total of at least sixty in the ampholyte mixture (LKB Ampholine pH 3-10, lot #17). Fifteen to twenty components were found in each 2 mm slice of a sequence of six slices of polyacrylamide gel subsequent to isoelectric focusing of sperm whale myoglobin with the same ampholyte mixture.

An ampholyte mixture prepared by the copolymerization of triethylene tetramine with acrylic acid was focused in the thin layer Sephadex gel on 20 x 5 cm plates and the eluates obtained from about 5 slices were analyzed by ion exchange chromatography. The results were compared with the caramelization pattern. The spatial distribution of individual ampholyte components were broad with half-band widths of approximately 1 cm.

The effects of sample load, ampholyte concentration and duration of focusing on spatial distribution were investigated in the PAG focusing of ^{14}C-histidine. The narrowest and most symmetrical distributions were obtained with small sample load and high ampholyte concentration. Even the sharpest distributions were much broader than given by proteins.

Background

Although staining (Frater, 1960) and caramelization (Felgenhauer and Pak, 1973) patterns and scanning photographs (Rigetti et al., 1975) have given some information about the distribution of ampholytes in isoelectric focusing, the high resolution and quantitative capability of the automated ninnydrin analysis procedure described previously should give a clearer description of the quantitative relationship of the various ampholyte components. Svensson (1962) considered the concentration distribution of an electrolyte as an equilibrium between electric mass transport and diffusional mass flow. Assuming a constant pH gradient and constant conductivity throughout a focused zone he obtained an analytical solution which describes the concentration distribution of ampholyte as Gaussian. It is well documented (eg., Righetti et al, 1975) that these assumptions do not hold in many systems and accordingly it seemed worthwhile to examine ampholyte distribution in gel and Sephadex thin layer systems.

Procedure

Isoelectric focusing was performed in 6 x 75 mm PAG (6% total) crosslinked with methylene bisacrylamide (3.0% C crosslinked) and polymerized using 0.1% (w/v) N,N,N^{1}N^1-tetramethylene diamine and 0.075% (w/v) ammonium persulfate. The gels were prefocused for

1.5 hr. An ampholyte concentration of 2% (w/v) was used unless otherwise stated. The ampholyte used in the gel experiments was LKB lot #17 (pH 3-10). After the samples were applied the current was maintained at 1 ma per tube until a voltage of 150 v was reached. Focusing was carried out for 20 hr at 4° C, using a Buchler Polyanalyst apparatus. The anolyte and catholyte were 0.01 M H_3PO_4 and 0.02 M NaOH, respectively.

Two types of experiments were performed. In one, sperm whale myoglobin (Mann Res. Labs.) and β-lactoglobulin (Pentex, lot #34) were focused separately, employing loads of 15 µg and 60 µg per gel, respectively. Upon completion of focusing the gels were removed from their tubes and frozen immediately in liquid nitrogen. This process required about 45 sec to 1 min. The frozen gels from the β-lactoglobulin experiment were than sliced manually. In the case of β-lactoglobulin one slice of about 1 mm was cut from between the two precipitated components. In the case of myoglobin, six slices of 2 mm were cut starting with the protein band and proceeding in the direction of the anode. The slices were extracted in 1 ml of water for 2 days at room temperature. In the case of β-lactoglobulin, the slices from three gels were extracted together. The extract was flash evaporated, brought up in 0.25 ml of pH 2.7 citrate buffer and analyzed by ion-exchange chromatography using the automated ninhydrin procedure.

In another set of experiments different amounts of histidine containing about 0.04 µg of ^{14}C-histidine were focused on PAG as described above for various times. The gels were frozen immediately after removal from the tubes with powdered dry ice and sliced into 1 mm slices with a Mickle gel slicer. The gel slices were incubated with 0.25 ml of 5% w/v water protosol mixture for 3 hr at 60°. After this 10 ml of scintillation solution (16 g PPO, 0.4 g POPOP diluted to 1900 ml with toluene) was added to each gel slice and they were counted in a Packard Model 3375 liquid scintillation spectrometer. The counts obtained from 2 or more 5-minute periods were averaged. The ^{14}C-histidine was obtained from the New England Nuclear Company and had a specific activity of 2 mCi/mg.

Isoelectric focusing was also done on thin layer Sephadex gels. The gels were prepared by spreading a slurry of 3.25 g of dry superfine Sephadex G-75 in 50 ml of solution containing 0.4 g of ampholyte on a 20 x 5 cm glass plate. The separation was carried out at 200 volts for 3 hr followed by 3 hr at 300 volts and finished with 500 volts for 1 hr. A caramelization pattern was obtained and the gel cut into 5 mm sections. For ninhydrin analysis the Sephadex fractions were eluted with 5 ml of water. An aliquot of 0.3 ml was analyzed.

Results

Location of Ampholytes in Polyacrylamide Gel Focusing. The two genetic variants of β-lactoglobulin, A and B, possess isoelectric points at 5.21 and 5.34, respectively (Kaplan and Foster, 1971). They are completely resolved by PAG isoelectric focusing. The gel

slice about 1 mm thick between the two precipitated bands was cut out, extracted with water and analyzed (Fig. 6). Although a major component was present, appreciable amounts of other ampholyte components were found. A total of 12-14 components were observed. These cover a fairly wide area of the chromatogram of the complete ampholyte mixture.

In another series of experiments using PAG focusing, we analyzed the ampholytes focused in six sequential slices, each 2 mm wide, using myoglobin as a marker protein. Analysis of the slices (Fig. 7) showed that each of the slices contained 15 to 20 ampholyte components. Gels frozen 2 and 5 min after completion had identical analyses with those frozen within 45 sec. Components with identical chromatographic behavior appeared in several slices. The width occupied by a given ampholyte component was wider than one would expect from the sharpness of the protein bands.

Spatial Distribution of Ampholytes in Thin Layer Sephadex Gels. Because the ion-exchange chromatographic elution patterns obtained with commercial ampholyte mixture are complex due to the large number (over 60) of components present, it is not possible to readily follow the concentration of a given component in successive slices. To avoid this problem an ampholyte prepared by the copolymerization of acrylic acid with triethylenetetramine (TETA) was used. Our preparation possessed a relatively small number of well resolved components which could be identified by their ion-exchange chromatographic behavior (Fig. 3). This ampholyte mixture was focused in a thin layer of Sephadex G-75. The resultant pH gradient and caramelization pattern obtained by the procedure of Felgenhauer and Pak (1973) is shown in Fig. 4. Ion exchange chromatography and ninhydrin analysis of fifteen of the thirty-eight fractions obtained by Sephadex thin layer isoelectric focusing are given in Table I and the concentration of certain components is plotted in Fig. 8. The results obtained permit a direct comparison with the caramelization pattern. The individual components are again distributed into several fractions. Although the number of fractions taken across a given ampholyte peak is rather small, the distributions of some of the components are asymmetric. It is also evident that the caramelization reaction does not correlate well with the ninhydrin color.

Distribution of ^{14}C-Histidine in Polyacrylamide Gel Focusing. Isoelectric focusing of ^{14}C-labeled histidine was used to obtain information about the behavior of a single low molecular weight amphoteric substance. Mixtures consisting of various amounts of carrier histidine added to a constant amount of ^{14}C-histidine were focused and the distribution of radioactivity was determined. When labeled histidine alone was focused a single, sharp, symmetric peak was obtained (Fig. 9). This peak became flatter and more asymmetric with increase in histidine concentration.

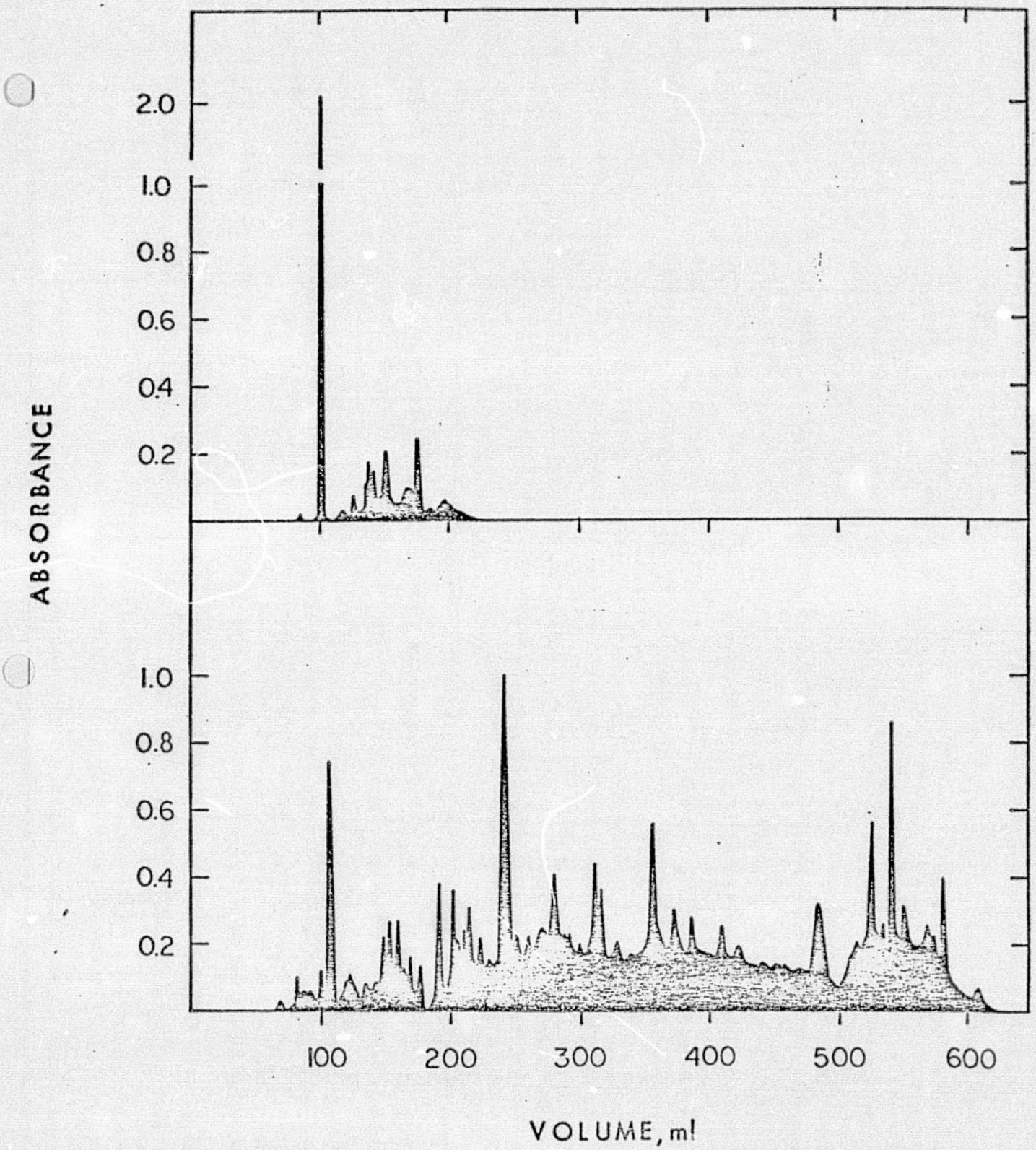


Figure 6. Analysis of the eluate from the PAG slice between the β-lactoglobulins A and B and of the LKP Ampholine (lot #17, pH 3-10). The absorbance is plotted against elution volume. The baseline obtained in a blank run was subtracted in both cases.

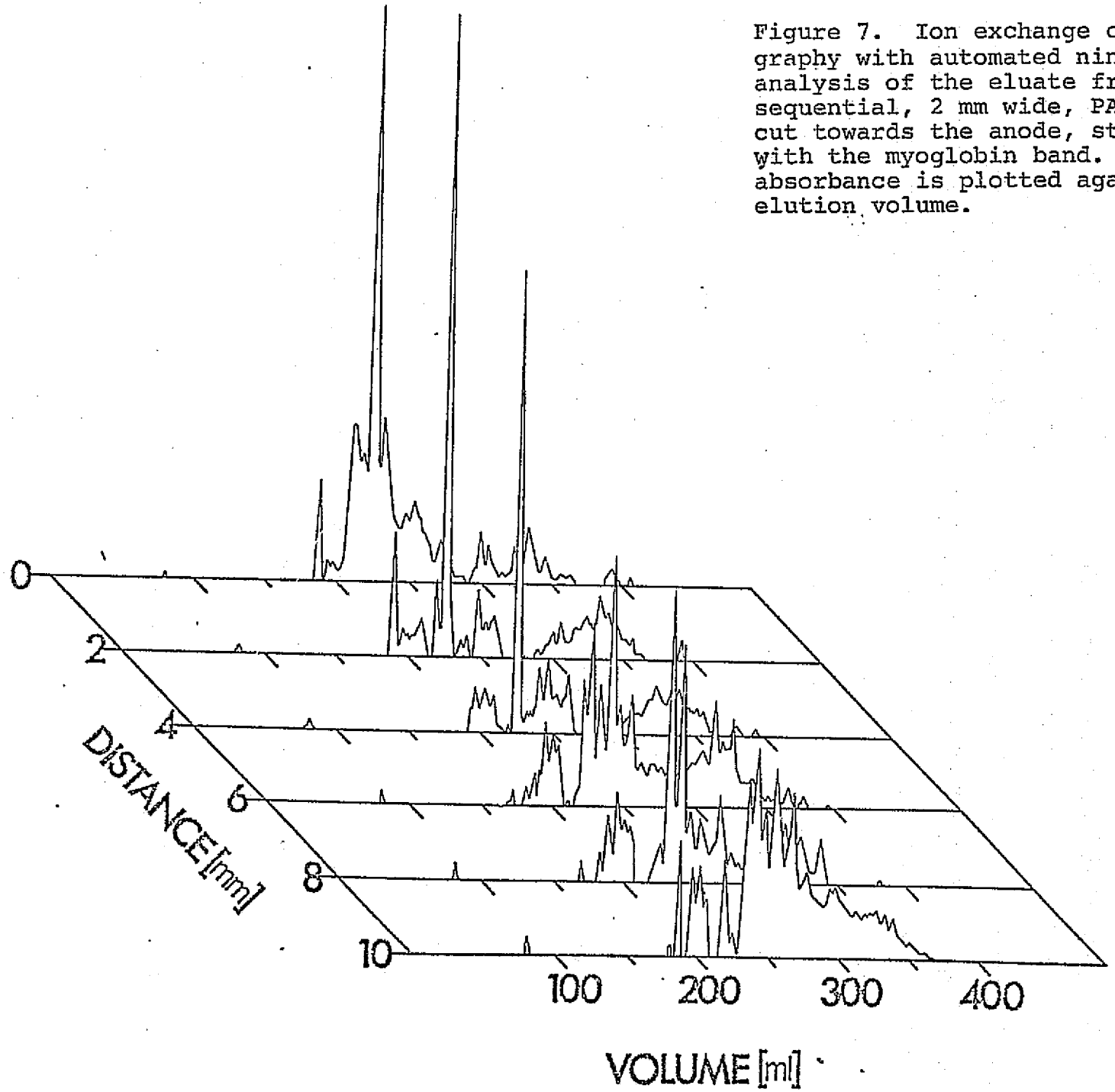


Figure 7. Ion exchange chromatography with automated ninhydrin analysis of the eluate from six sequential, 2 mm wide, PAG slices cut towards the anode, starting with the myoglobin band. The absorbance is plotted against the elution volume.

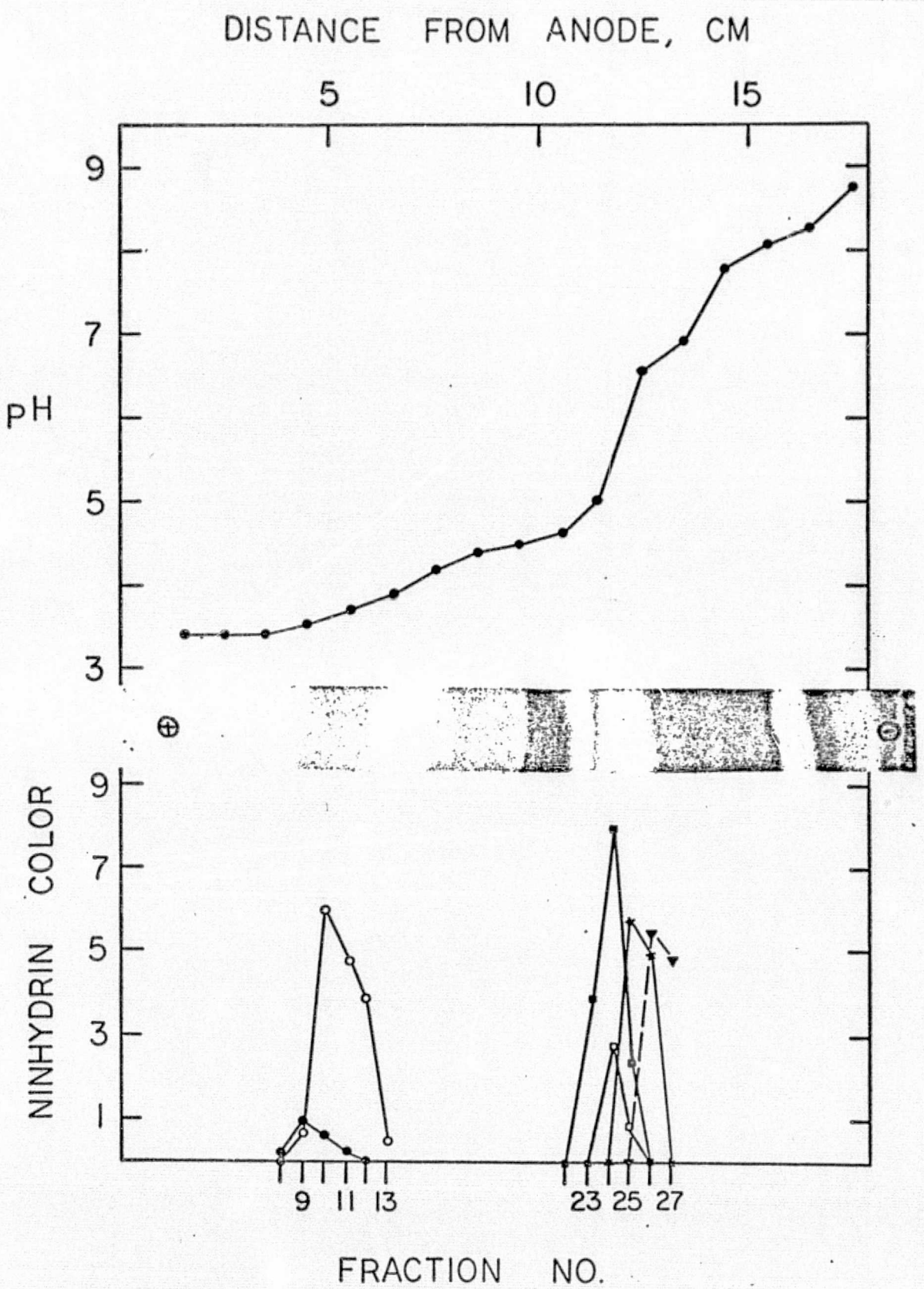


Figure 8. The pH gradient, caramelization pattern and the distribution of some of the ampholyte components after isoelectric focusing of the TETA ampholyte preparation in a thin layer of Sephadex G-75. Individual ampholyte components are identified by their elution volume in the ion-exchange chromatography: • = 48 ml; ○ = 63 ml; ■ = 101 ml; □ = 184 ml; ★ = 201 ml; ▼ = 235 ml. Each fraction corresponds to about 5 mm of gel layer. EAch point in the lower portion represents the sum of the absorbances under each peak of the foregoing ampholyte components in the analyses of the individual fractions.

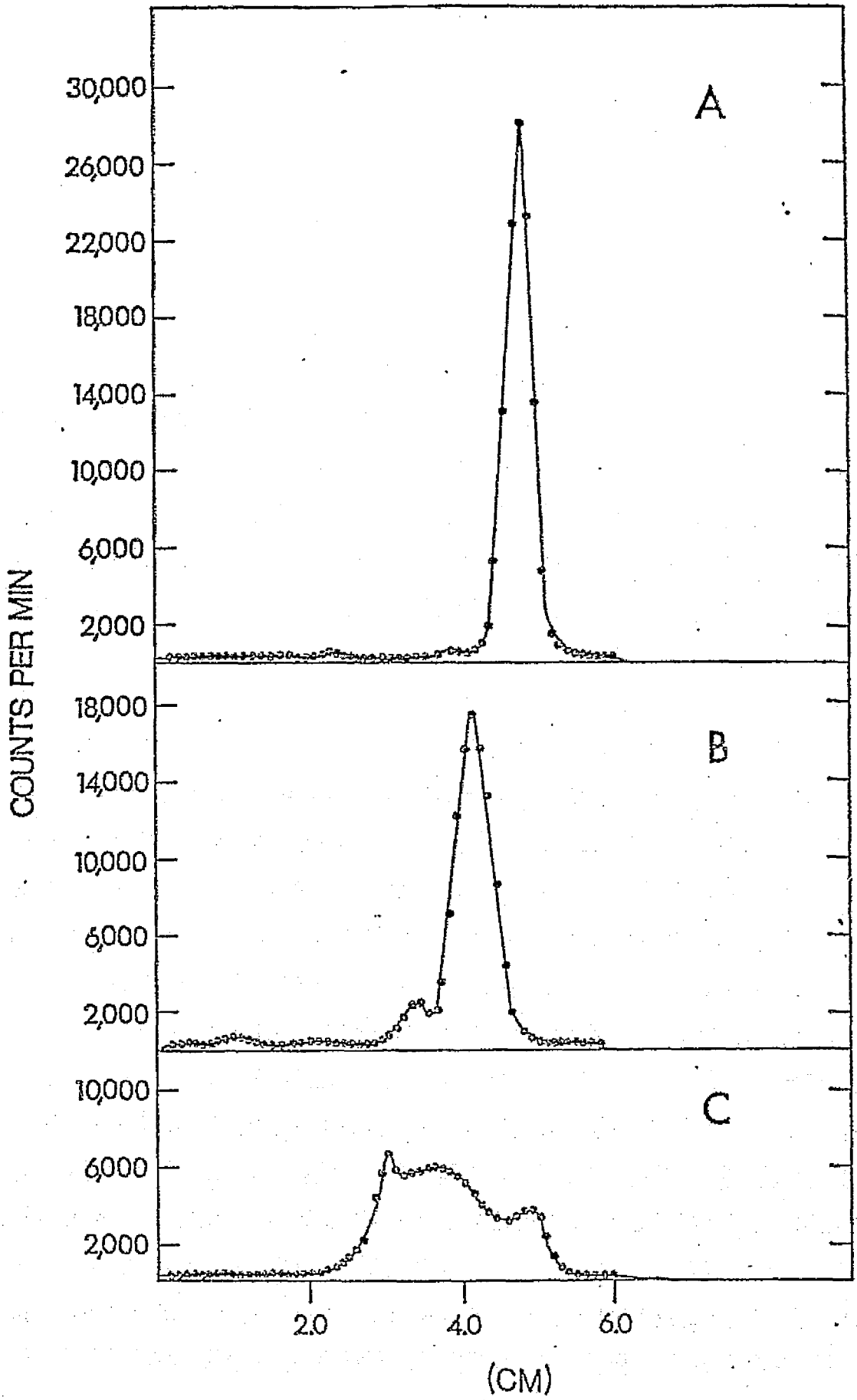


Figure 9. The effect of total histidine concentration on the distribution of 0.04 mg of ^{14}C-histidine during isoelectric focusing in PAG. The radioactivity is plotted against the distance from the anode. The total amount of histidine used per ml of gel was 0.04 µg (A), 0.8 mg (B) and 5.1 mg (C).

Table I

ION-EXCHANGE CHROMATOGRAPHIC ANALYSIS OF FRACTIONS OBTAINED

BY TLSG ISOELECTRIC FOCUSING OF TETA AMPHOLYTES

Elution Volume, ml	Percent of total ninhydrin color	Percent of total ninhydrin color of each ampholyte component applied to TLSG recovered in each fraction														
		Fraction Number[a]														
		8	9	10	11	12	13	21	22	23	24	25	26	27	34	3
48	.54	.03	.18		.03											
63	4.01			1.08	.85	.71	.09									
76	.33					.06										
101 }	6.88									.69	1.38	.42				
114 }								.45	.45							
130	16.07							.16								
152	1.63							.06	.09	.16						
184	1.99										1.46	.49	.13			
190	6.16					.02	.06									
196 }	6.93								.05	1.04	.75					
201 }												.89	.77	.08	.42	
235	14.05											.06	3.51	2.70	3.73	
256	6.53															
265	10.60													1.71		
296	.70														.08	.1
367	5.01														2.21	
391	1.17															
411	2.37															.0
449	2.40															.8
473	7.48															.7

[a]Each fraction corresponds to about 5 mm of thin layer gel

The duration of isoelectric focusing was varied in order to determine whether it affected the distribution observed at high histidine concentrations. The results (Fig. 10) showed that there was little difference, other than a slight cathodal migration, between focusing for 18 and for 24 hr. After 42 hr the histidine peak was further flattened and shifted towards the cathode.

The effect of ampholyte concentration upon the distribution of histidine was examined by running a constant amount of histidine (0.04 µg ^{14}C-histidine plus 0.8 mg carrier histidine) in the presence of 4%, 2% and 0.2% ampholyte (Fig. 11). Under the conditions used the histidine focused most sharply at the high ampholyte concentration used.

D. PREPARATIVE ISOELECTRIC FOCUSING IN NON-LINEAR pH GRADIENTS

Insufficient separation between components has been a major problem with isoelectric focusing. Components which separate under analytical conditions involving 10 or 20 µg of protein tend to overlap when loads of 10-20 mg are employed. Deformation of the separated zones by convective and endosmotic effects adds to the separation difficulty. The studies in the previous section of this report suggest a means of increasing the separation distance between specific ampholyte components. This has been studied with antibodies to oxidized ribonuclease and in preliminary experiments with human gamma globulin, a bacterial amylase and intestinal phosphatase. In the case of antibodies it has been possible to find single ampholytes which distribute on both sides of that antibody giving a single well separated antibody band.

When the amount of ampholyte is held constant addition of an amphoteric substance flattens the pH gradient. The extent of the flattened portion is proportional to the amount of amphoteric substance added. This effect occurs both with polyacrylamide gels and with preparative isoelectric focusing in sheets of superfine Sephadex G-75 (Fig. 12). There is still a pH gradient in the gels and by proper choice of amphoteric substance it is possible to have the flat portion of the gradient on both sides of the substance to be separated giving it wide separation from substances even slightly acid or alkaline to its isoelectric point.

The pH of the center of the flattened portion approximates the pI of the added amphoteric substance (Fig. 13). In certain pH ranges appreciable numbers of amphoteric substances are available commercially (Table II) but for other ranges it will be necessary to synthesize materials. The studies of the synthesis of ampholytes and their analysis by ion exchange chromatography provide an approach which is currently being studied.

Some preliminary experiments with methemoglobin prepared from a single individual indicate the separations achieved and the effect of added amphoteric substances on the separation (Fig. 14).

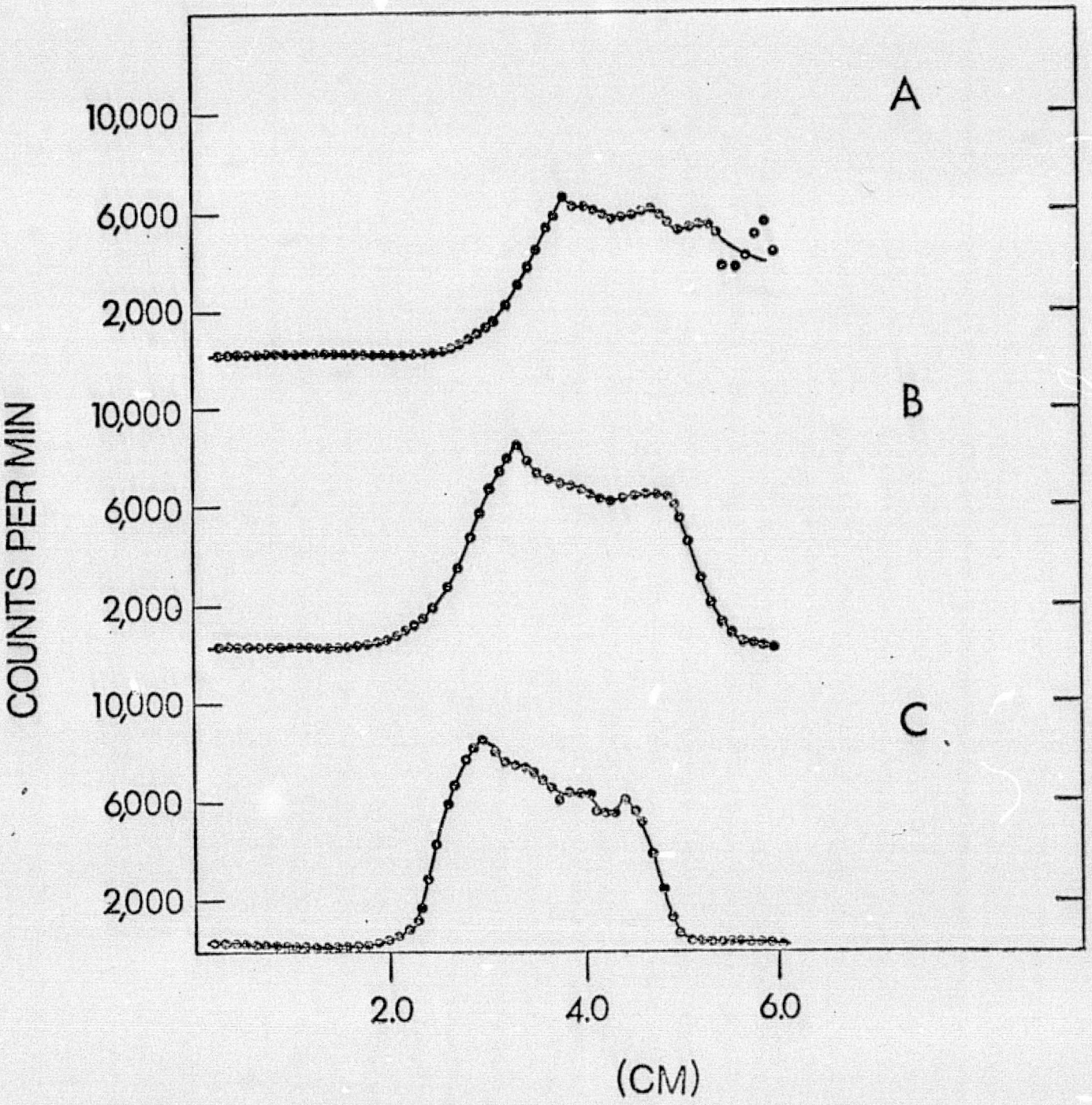


Figure 10. The effect of duration of focusing in PAG upon the distribution of ^{14}C-histidine. Histidine 5.1 mg/ml was focused in 2% LKP Ampholine (Lot #17) for (A) 18, (B) 24 and (C) 42 hr.

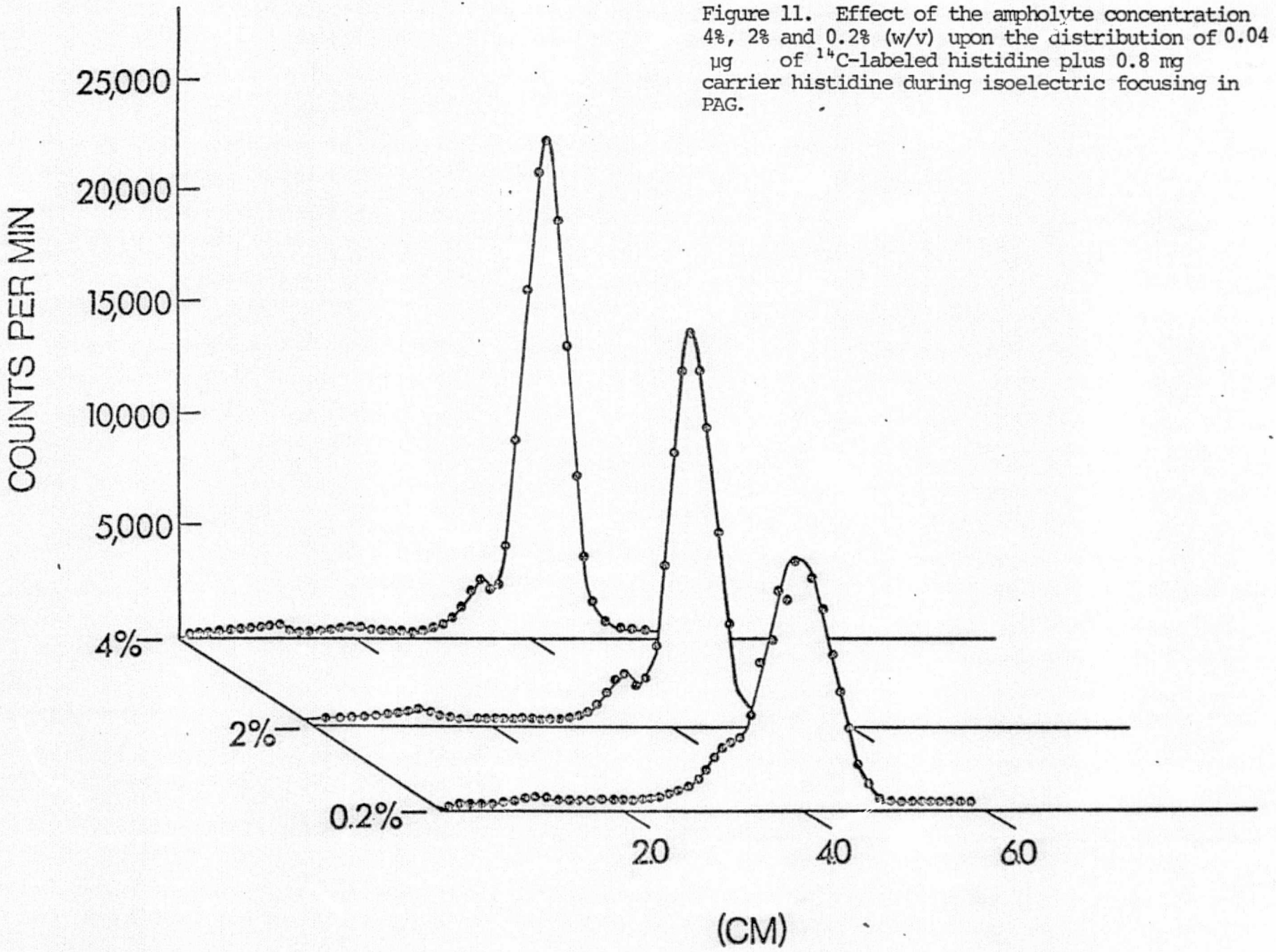


Figure 11. Effect of the ampholyte concentration 4%, 2% and 0.2% (w/v) upon the distribution of 0.04 µg of ^{14}C-labeled histidine plus 0.8 mg carrier histidine during isoelectric focusing in PAG.

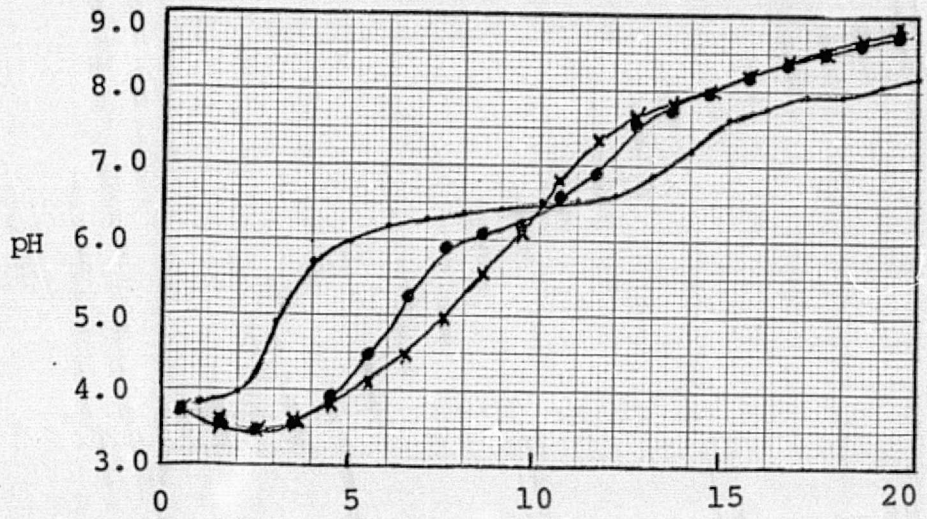


Fig. 12. Effect of adding β-alanine to the ampholyte used in thin layer isoelectric focusing. X = ampholyte alone; • = ampholyte + 0.6% β-alanine; ●—● = ampholyte + 6% β-alanine.

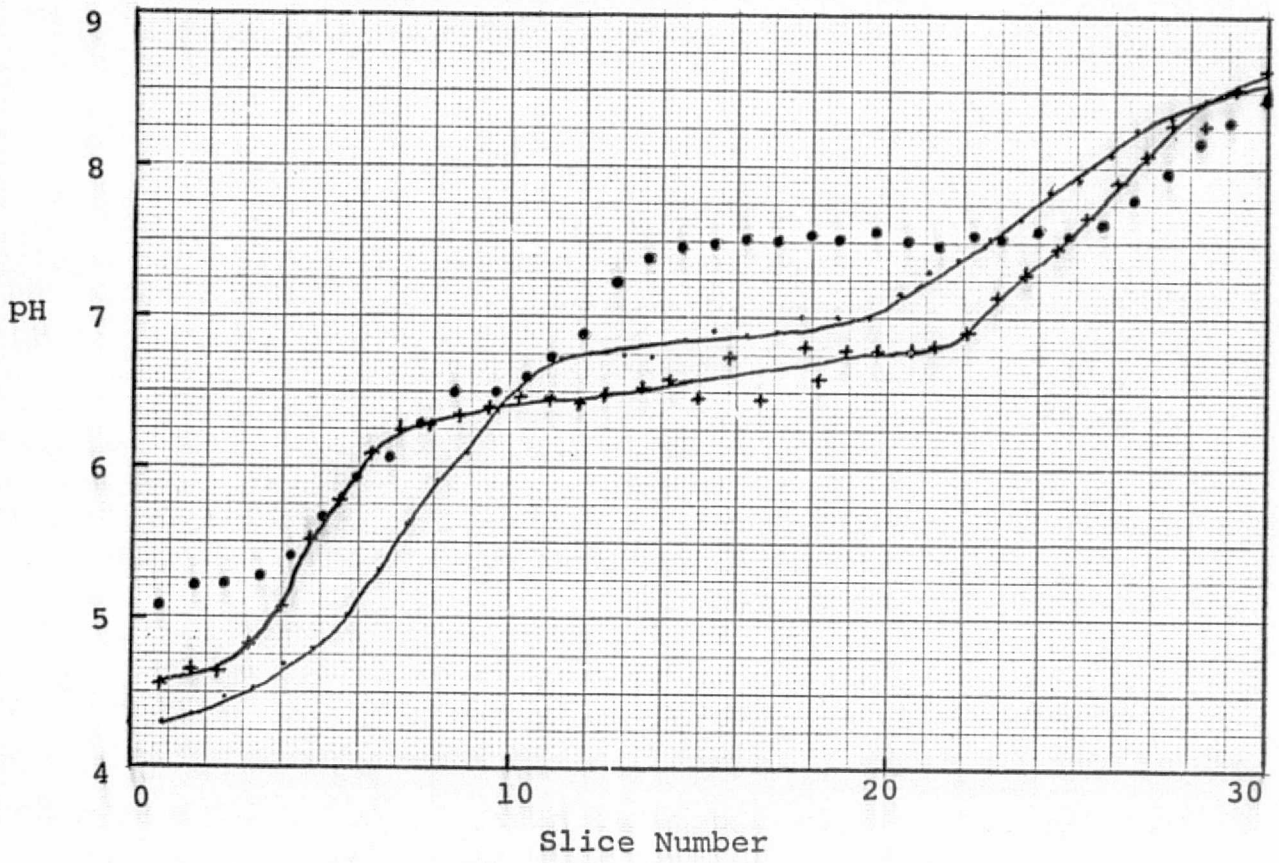


Fig. 13. Effect of added amphoteric substances on pH gradient in acrylamide gels.

• = histidine · = ε-aminocaproic acid X = β-alanine

Table II

pI OF CERTAIN AVAILABLE AMPHOLYTES IN THE pH 5.59 to 6.16 Range

pI	Compound	pI	Compound
5.59	Glycyl glycyl glycine	5.85	Isoglutamate
5.60	Glycyl glycine	5.89	Tryptophan
5.61	Allothreonine	5.90	N-dimethyl glycine
5.62	Glycyl alanyl alanyl glycine	5.92	Citrulline
5.64	Threonine	5.94	Isoleucine
5.65	Glutamine	5.96	Valine
5.66	Tyrosine	5.97	Glycine
5.68	Serine	5.98	Leucine
5.69	Glycyl proline	6.00	Alanine
5.70	Glycyl alanine	6.02	Isoserine
5.71	Glycyl valine	6.04	α-amino valeric acid
5.72	Alanyl alanine	6.08	α-amino butyric acid
5.73	Glycyl leucine	6.10	Lysyl glutamic acid
5.74	Hydroxyproline	6.12	Sarcosine
5.79	Allohydroxyproline	6.15	Hydroxyvaline
5.80	Sarcosyl glycine	6.16	Oxy-α-aminobutyric acid
5.83	Oxyproline		

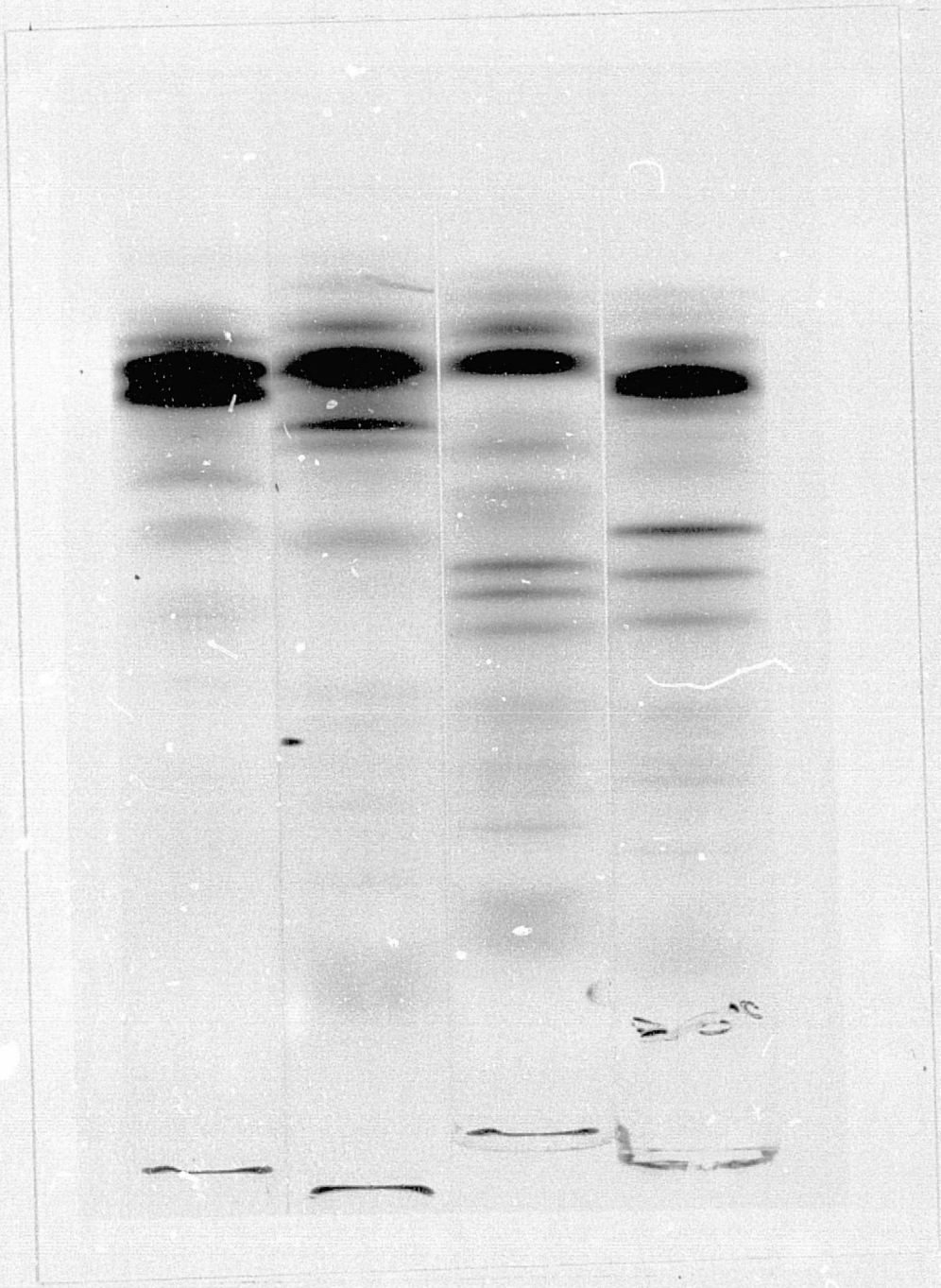


Figure 14. Effect of adding various amphoteric substances for the isoelectric focusing of human methemoglobin. A - no addition; B - 4 aminobutyric acid (10 mg/ml); C- 6 amino caproic acid (10 mg/ml; D - 5 amino valeric acid (10 mg/ml).

VI. FACTORS AFFECTING THE STABILITY OF ANTIBODIES

Summary

The stability of antibody was examined by determining the amount of goat gamma globulin remaining in solution after storage under a variety of conditions of pH, temperature, and ionic strength. The effects of stabilizers and of freezing and thawing were examined. The antibody studies is reasonably stable between pH 4.0 and 9.0 if kept cold. At neutral pH it is stable at room temperature. An ionic strength of 0.001 at pH 7 seemed borderline for solubility. Glycerol deserves further study as a stabilizing agent. The retention of biological activity was determined at pH 4.5 at 0° and 24°. Over 50% of the activity was lost in 47 hr at 0°. Freezing and thawing were quite deleterious under the conditions used. While 60-90% of the material remained soluble after storage at -18° for 20 days an additional freeze-thaw cycle left only 3% in solution. Many variables remain to be more carefully explored. Caution should be employed in extrapolating these observations to other immune systems.

Introduction

Human gamma globulin preparations are usually stored as a 10% to 18% solution containing 0.3 molar glycine and 0.01% thimerosal (merthiolate). When stored at +5° such products have a shelf life of 3 years. Since they may be held for 3 years before release the actual storage life is 6 years. Since the antibodies for the proposed separation at low gravity are from goats and the proposed separation conditions are quite different from those above, a study was made to assess factors influencing antibody stability.

Proteolysis by plasmin is believed to cause most of the degradation found in samples of human gamma globulin stored for prolonged periods. Attempts to predict stability by short term incubation for one month at 20° (Painter and Minta, 1969) have been unsuccessful but procedures in which the plasminogen is activated before the test show promise. Procedures assaying proteinase activity have little predictive value. Proteolysis of antibody is apt in general to be a slow event which is not an important factor in the kinds of short term experiments proposed here. Accordingly our attention has been concentrated on physical and chemical factors with more immediate effects. In general the several variables that must be tested simultaneously each have an effect on the outcome.

Temperature is a fairly critical variable when other conditions are borderline. Thus, antibody at pH 4.5 which is near the lowest pH where it is stable is much better preserved at 0° than at 24°. The solution contained 2.5 mg of precipitable antibody and 6.9 mg of total protein. It was stored in pH 4.5 acetate buffer which was 1 molar in the Na^+.

After the appropriate time interval the samples were placed on a small, 10 ml, column of Sephadex G15 equilibrated with phosphate (.05 m) buffered saline (.1 m), pH 7.4 maintained at the temperature of the experiment. Fractions were collected and the two with the highest concentration of proteins were assayed by precipitin analysis. Seven concentrations of antigen all near equivalence were used for each assay. This procedure allowed us to examine the storage step per se rather than having to perform an additional concentration step.

The recovery of total protein in the procedure was within experimental error the same in all specimens indicating that little gross denaturation occurred during the period. The immunologic activity declined quickly in the samples stored at 24° while only modest but discernable loss was encountered in the samples stored at 0° (Table III). The results are expressed as fraction of total protein which was precipitable with antigen at the point of maximum precipitation (equivalence point) of the precipitin curve.

These studies suggest that storage for even a few hours at pH 4.5 and room temperature is quite deleterious. If the storage is carried out at 0° the antibody survives much better but after two days under these conditions some inactivation occurs.

The effect of temperature upon the storage of normal goat gamma globulin (0.4%) was studied by observing the optical density of the material remaining in solution after centrifugation. The pH of the material was 7.2, the solution was 0.1 m in NaCl, 0.05 m in phosphate and 0.02% in sodium azide. Solutions kept at room temperature and at 4° C were observed over the next 85 days (Fig. 15). Little change was observed although there is a small increase in the absorbancy of the material which was stored at room temperature. Aggregation to form pauci-molecular complexes which increase the turbidity of the material is the probable reason for the small increase in optical density observed. Some additional inferences regarding the effect of temperature at low pH can be drawn from immunoabsorption studies described in a later section.

Freezing under the conditions employed was quite deleterious. The conditions were as above, 0.4% goat gamma globulin, pH 7.2, 0.1 m NaCl, 0.05 m sodium phosphate, 0.02% sodium azide. After storage for 20 days at -18°, 66% of a sample frozen in a plastic tube remained soluble. Ninety-one percent of a similar sample in glass was soluble. After the samples were frozen and thawed again, only about 3% of the material was soluble. We set the material in the -18° room to freeze it and thawed it in a beaker of water at room temperature. It seems probable that better conditions for survival during freezing and thawing could be developed. Human gamma globulin is freeze dried during its preparation and probably survives because of the higher protein concentration.

Table III

EFFECT OF STORAGE ON GOAT ANTIBODY AT pH 4.5

Time (hr)	Temperature (C°)	Antibody/ Total Protein
0	0	.36
6.2	0	.35
47.	0	.31
0	24	.35
3.2	24	.17
6.8	24	.15

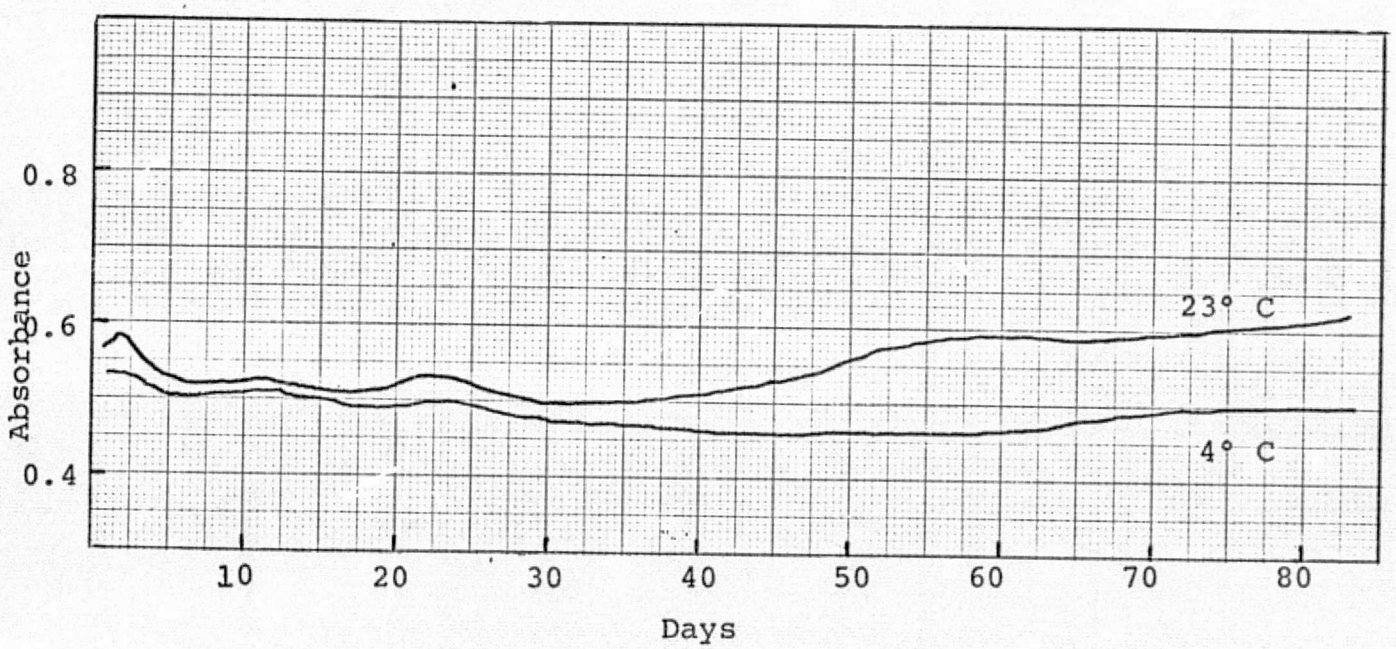


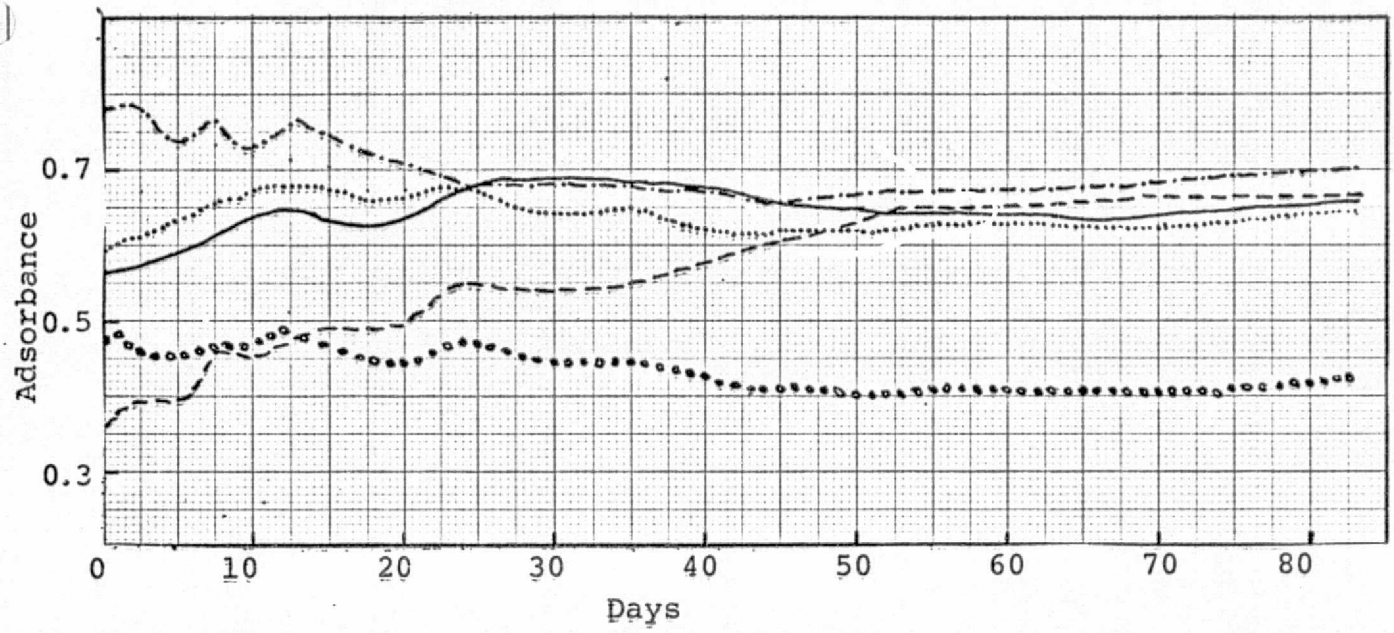
Fig. 15. Effect of storage temperature on gamma globulin.

The work of Curti et al. (1968) is an elegant demonstration of the role of the conditions used for freezing and storage in the inactivation of the enzyme L-amino acid oxidase. We have done immunological studies of the conformational changes observed upon the inactivation of the enzyme which occurs on freezing and of its reactivation (Zimmerman et al., 1971). It seems likely that studies of freezing temperature, freezing rate, thawing conditions, protein concentration, and perhaps the use of additives would provide conditions where the material would survive the two cycles of freezing and thawing proposed for preliminary experiments. It is also possible that renaturing conditions could be found.

pH effects were studied on goat gamma globulin to access general stability of the protein. In each the protein concentration was 0.4%, 0.15 m NaCl, 0.075 m buffer ions and 0.02% azide. Storage was at +4°. Samples were examined 2 or 3 times per week over an 83-day interval. Sodium acetate buffers were used for pH 4 and 5, potassium phosphate for pH 6 and 7 and tris acetate for pH 8 and 9. Little change in solubility occurred under these conditions (Fig. 16). Other pH studies include those reported under the temperature heading where antibody activity was examined. The decline in activity can be contrasted with samples kept at neutral pH in the cold where the activity is unchanged after many months of storage. The effect of pH on the assay of antibody is discussed in a later section of this report.

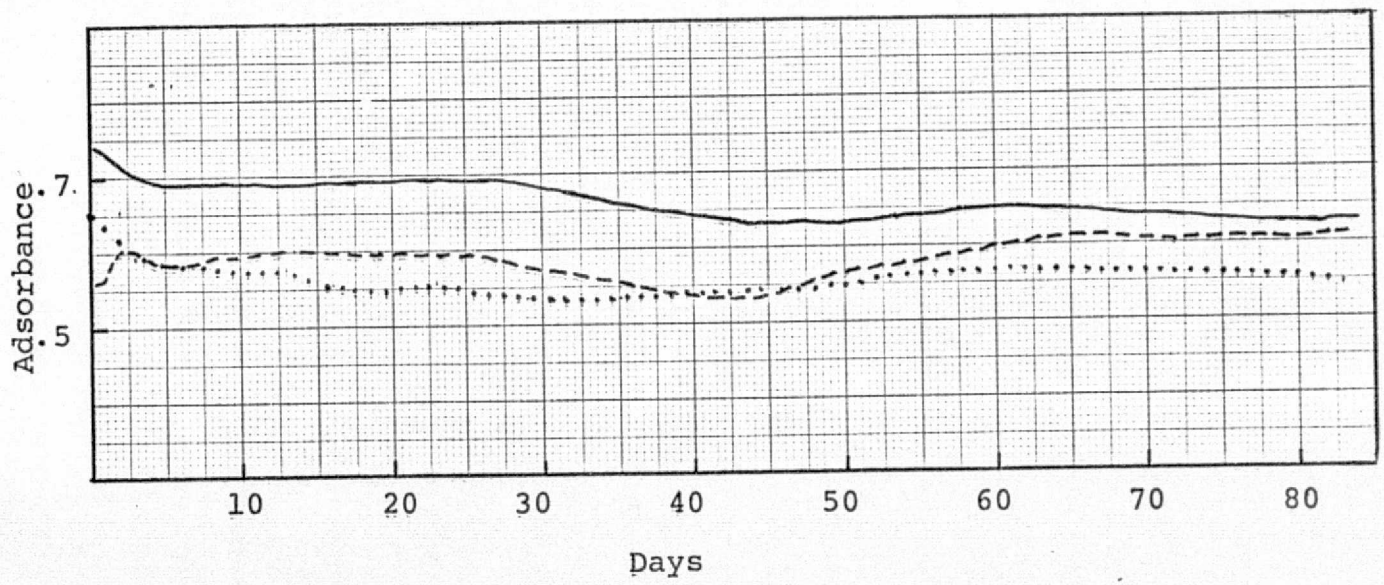
Ionic strength is of significance in that antibodies are in large part euglobolins which are insoluble in the absence of salt at their isoelectric point. This insolubility is especially troublesome during isoelectric focusing in liquid systems and is a major reason why processing at zero gravity may be of especial importance in their purification. The stability studies undertaken were primarily to determine the lower limits of ionic strength suitable for electrophoresis. They were at 0.4% goat gamma globulin, 4° C, pH 7.0, 0.02% sodium azide. The buffer was potassium phosphate 0.001, 0.01, 0.1 or 1.0 m. A set of experiments which were 0.15 m in NaCl was also run. All samples maintained good solubility although about 14% of the material stored in 0.001 m potassium phosphate became insoluble during the course of the experiment (Fig. 17). This probably is close to the lowest ionic strength at which the material will remain soluble. It is obvious that changing the pH away from the isoelectric point in either direction would improve solubility.

Stabilizers were also tested. Glycine 2.25%, sucrose 10% or glycerol 10% were tested under the conditions used previously (0.4% protein, 0.10 m NaCl, pH 7.2, 0.05 m sodium phosphate, 0.02% sodium azide). The glycine concentration is that used for storage of human gamma globulin. Since little change was seen in the controls it is difficult to determine whether any of the substances had much effect. Glycerol stored samples maintained the most constant absorbance. There was marginal increase in the absorbance of the samples stored in glycine or sucrose.



ooooo = pH 4.0; -·-·-·- = pH 5.0; ----- = pH 7.0; ····· = pH 8.0; _____ = pH 9.0
Material stored at pH 6.0 behaved similarly to that at pH 5.0.

Fig. 16. Effect of pH on gamma globulin



····· = .001 m ~~~~~ = .01 m ------ = .1 m

Fig. 17. Effect of ionic strength on gamma globulin. All samples in potassium phosphate buffer pH 7.0.

III. METHODS FOR ASSAYING ANTIBODIES

Consideration and development of methods suitable for inglight evaluation of antibody separation and refinement of methods for ground based studies make up this section.

A. Radial Immunodiffusion - A process for evaluating antibody separations in flight

Summary

A radial immunodiffusion assay was developed for the assay of antibodies to specific small areas of antigen. Antigen or its peptide fragments coupled to a carrier are incorporated in an agar gel. Antibody is added to a well cut into the gel. If the volume of antibody is kept constant and the temperature controlled the antibody concentration can be determined to about ± 10% over a 35-fold range starting at 7 µg/15 µl. Gravity plays no detectable role in the assay which was shown to be specific for antibodies to different regions of the antigen. Peptides were prepared by solid state synthesis and carefully purified and characterized for use in the system.

Introduction

A number of procedures are available for the estimation of antigens which use diffusion of antigen and/or antibody in gels. These methods rely upon the formation of an immune precipitate when the proportions of antigen and antibody are proper. Methods which allow the reactants to diffuse from separate points (Ouchterlony procedure) are widely used qualitative tests but are difficult to quantitate. Methods in which one of the components is immobilized in the gel are more quantitative. In single radial immunodiffusion one component (usually antibody) is placed in the gel. This procedure, often called the Mancini (1965) technic is widely used for quantitation. For example, commercial kits using this principle are available for about 20 different human plasma proteins.

Procedure

We have examined the following modifications of the Mancini procedure which should make it useful for estimation of antibody to specific small regions of antigen. It should also be of value for identification of the antibody and for determining the amounts of contaminationg antibodies directed toward other portions of the antigen. In the procedure developed antigen is placed in the agar layer and antibody is placed in the well. We have studied the dependence of the process on agar concentration, antigen concentration, antibody concentration and time. At present we are able to detect 7 µg of antibody in a 15 µl sample. Preliminary studies indicate a linear relationship between the area of the halo which surrounds the antibody well and the concentraiton of antibody over a 35-fold range. Optimal conditions are 2% agar

(Difco purified) 1.4 µg of antigen per ml agar 2 days at room temperature. Plate washed with saline and stained with amido black. Indications are that an accuracy of 10% can be achieved and that 3% of impurity can be determined.

To allow determinations of the amount of antibody for each of several different determinants, to identify the material and to quantitate the total amount of antibody, a plate with several sections was proposed. The process would be used as follows.

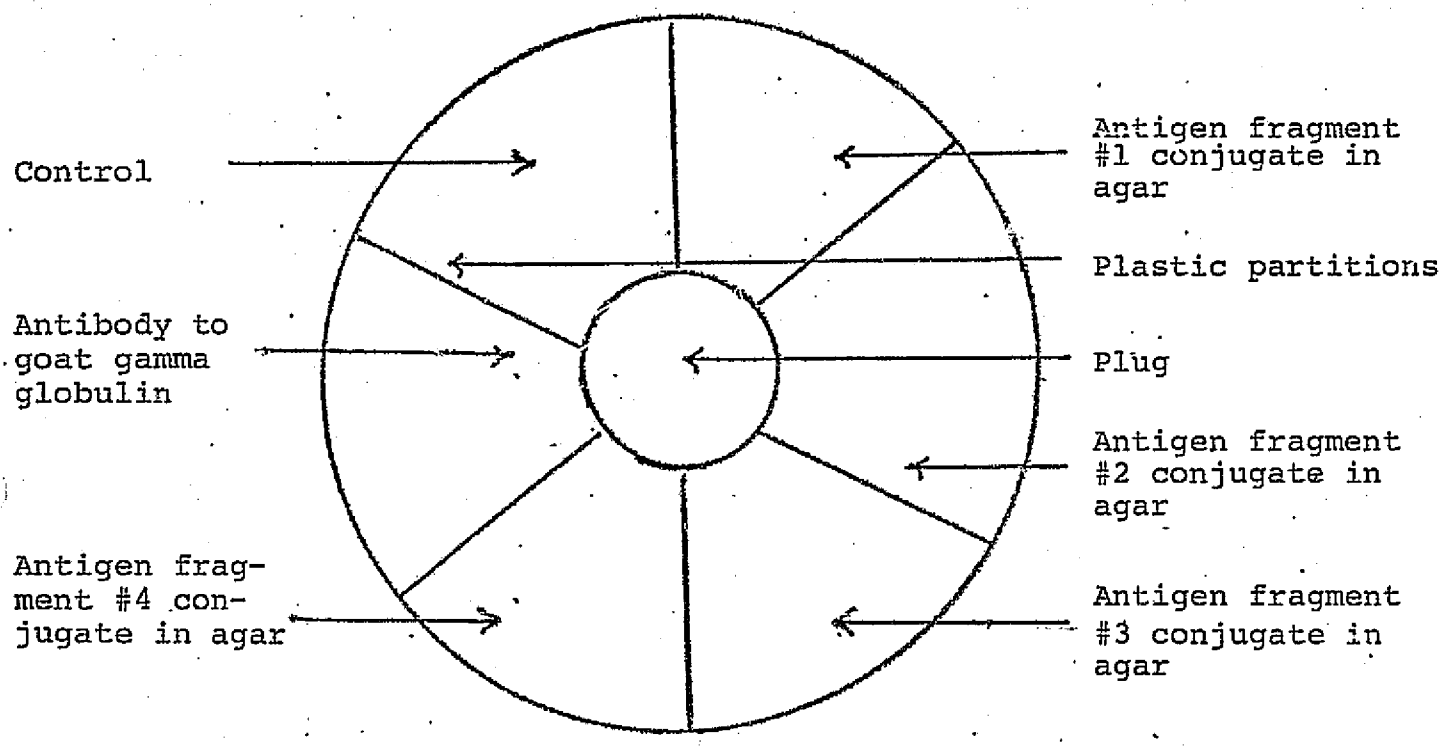


Fig. 18. Initial Setup

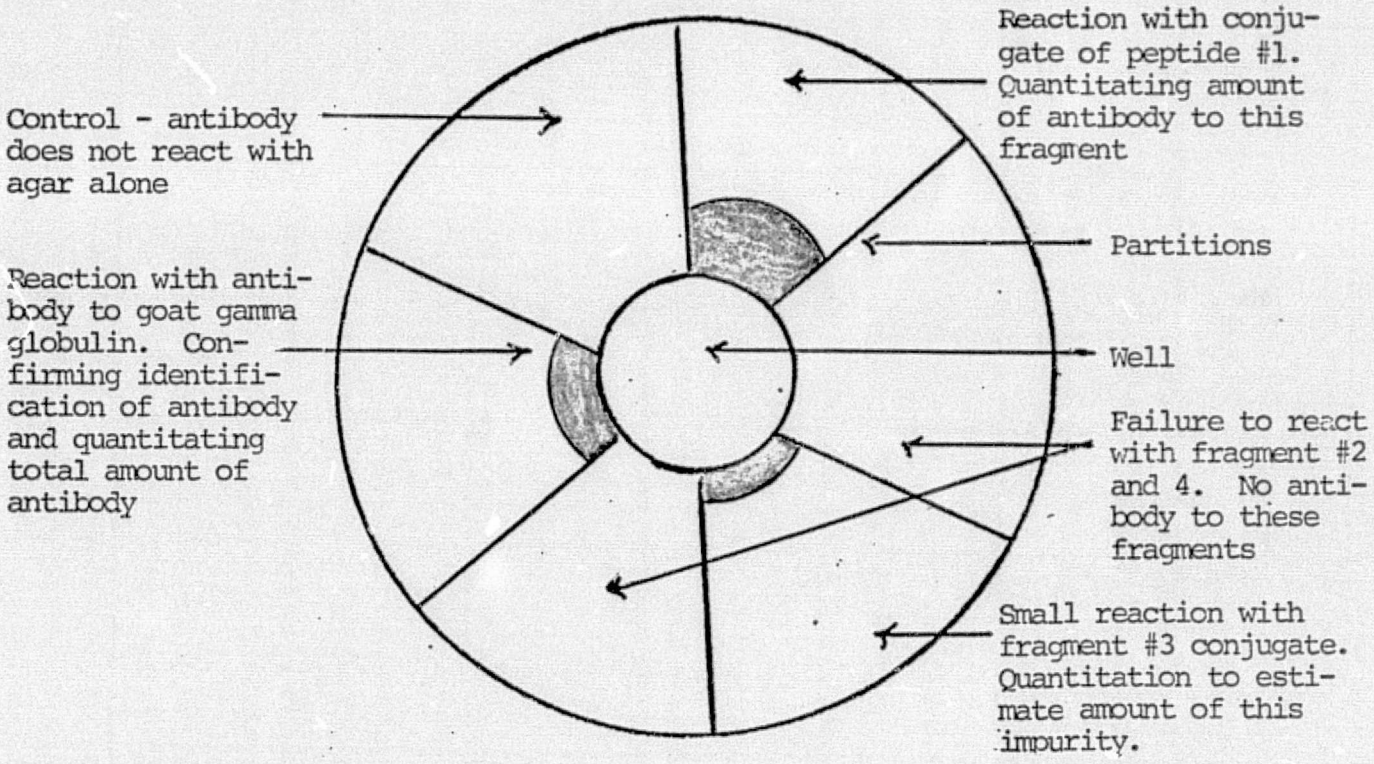


Fig. 19. Conclusion of Assay. Comparison of the amount of goat immunoglobulins with the amount of antibody to its specific fragment gives an index of its purity in biological terms.

Specificity

Tests were done for the specificity of the system by placing normal goat serum in the well. It gives a non-specific haze with a diffuse margin when bovine gamma globulin or oxidized ribonuclease is put in the agar. Normal goat gamma globulin gives no reaction under these conditions.

A variety of washing technics, 10% KCl, pH 7.7 borate buffer (0.5 m), pH 8.6 barbital buffer (0.1 m) and haemosol (0.2%) were all ineffective. Changing the type of agar was also of no avail. Current experiments are done with Difco Special Agar. Difco-Noble Agar (2%) and ion agar (1%) both gave hazy halos when tested with normal serum. The conjugates prepared with human serum albumin have not been testedbut may obviate the problem. The haze is not seen with the immunoglobulin preparations which are proposed for use in the separation and should cause no problems.

Effect of Volume

The volume of the test material must be kept constant. In one experiment 15 µl of a 1:4 dilution of sodium sulfate fractionated antibody was placed in each of 3 wells. Nothing was added to the first. Two 15 µl portions of saline phosphate buffer were added to the second and four 15 µl aliquots were added to the third. The diameters of the rings (including the well) were 0.6 cm, 0.8 cm and 1.1 cm. Thus, with a constant amount of total antibody in the test the diameter of the ring is almost doubled with a five-fold increase in volume. Accordingly, it is essential that the volume be kept constant.

Effect of Gravity

When the wells are placed too close together (less than 1.5 cm under the conditions we use) the halos around the well become ovoid (Fig. 20). When larger volumes are applied to wells further apart a similar effect may occur. We considered the possibility that this was due to gravitational mass fluid flow and became concerned about the role of gravity in radial immunodiffusion. Experiments in which immunodiffusion or simple diffusion patterns were set up with the glass slide on edge or upside down clearly indicate that gravity has no appreciable effect on the development of the pattern.

Assay of Antibodies to Specific Small Areas of the Antigen

In order to assay the various antibodies to the carboxy terminal region of oxidized ribonuclease (residues 105-124) it was necessary to prepare fragments of this region and others. This was done by enzymatic means or by synthesis as described in a subsequent section.

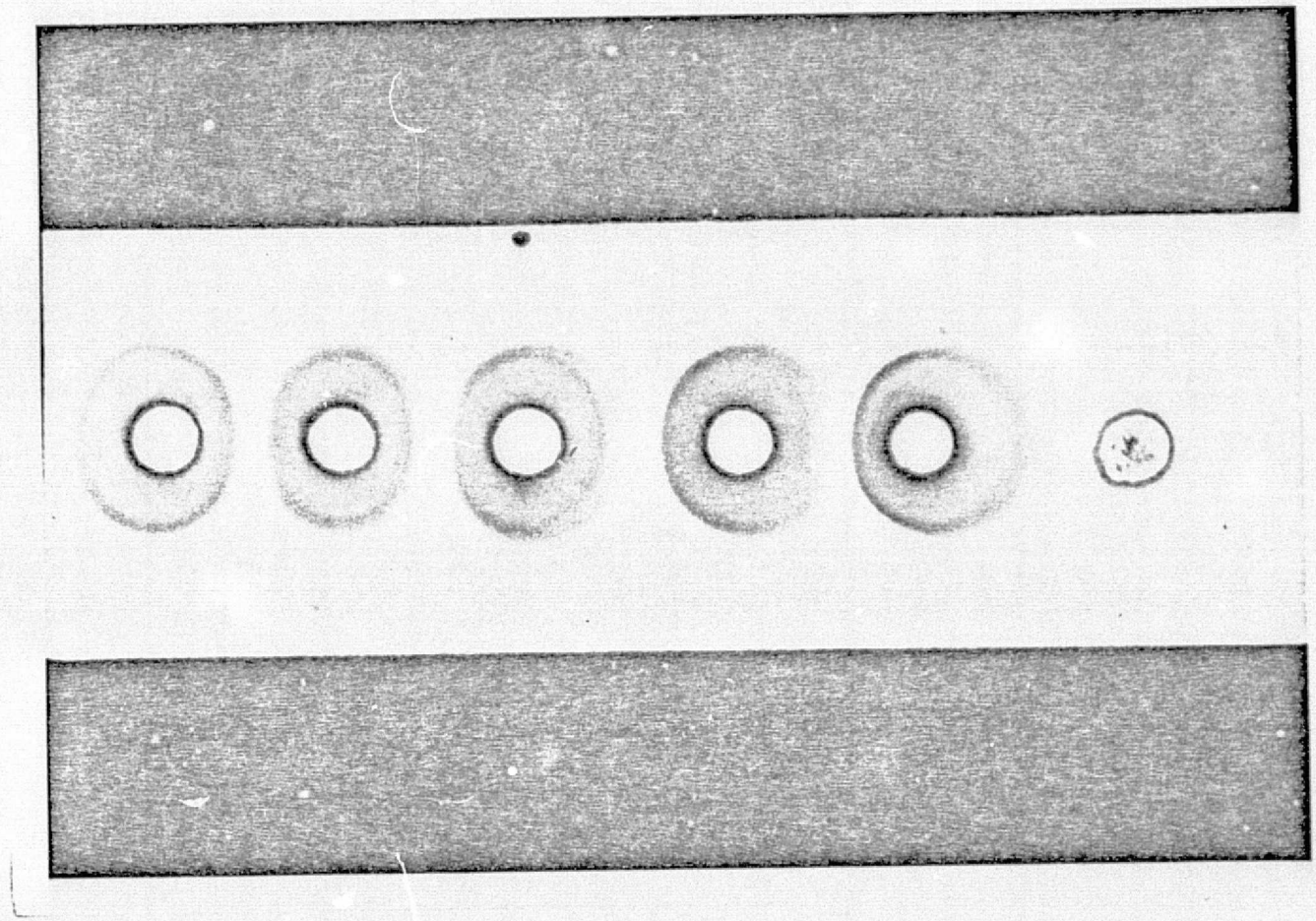
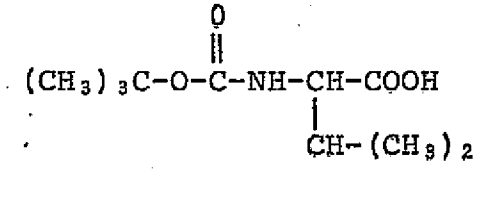


Fig. 20. Ovoid rings caused by placing wells too close together. Rightmost well had no antibody added to it.

The peptide conjugates were prepared by a modification of the procedure of Spragg et al. (1966). The peptide was activated in aqueous solution at 0° by addition of tolulene diisocyanate; 25 µl was added to 1.0 ml of solution. The reaction proceeded with continuous mixing on a shaker at 4° for 45 hr. The carrier, either bovine gamma globulin or human serum albumin, was dissolved in 1.0 ml of an aqueous sodium tetraborate solution, 90 mg/ml, and equilibrated at 45°. The activated peptide was added slowly to the carrier and care was taken not to transfer any activating reagent. The reaction was allowed to proceed at 45° for 2 hr with occasional mixing. Conjugates were separated from unreacted peptide and reagents at 4° by gel filtration on a 1 x 100 cm column of Sephadex G75 equilibrated with 0.05 m phosphate buffer pH 7.5 containing 0.1 m sodium chloride. The conjugate was located in the effluent by the Folin-Ciocalteau method. Serum albumin conjugates were more stable and allowed demonstration of the specificity of the assay procedure using antibody prepared by immunoabsorption. Antibody to peptide 105-124 reacted with peptide 105-124 conjugates but not those made with peptide 40-61. Antibody to peptide 40-61 reacted specifically with its conjugate. These tests further indicate the specificity and applicability of the process. Within each determinant it is not clear which regions react with which antibody band.

Synthesis and Characterization of Peptides

Chloromethylated polystyrene beads 1% cross linked were used as the insoluble matrix upon which the peptide is synthesized. The material was obtained commercially and had 1.1 meg of Cl per gram, i.e., about one CH_2Cl group per 10 styrenes. A 10 gram sample of resin was reacted with Boc-L-valine.



The Boc protecting group ($(CH_3)C-OC-$) was removed by treating with 20% trifluoroacetic acid (F_3CCOOH) in methylene chloride (CH_2Cl_2) for a two minute period followed by another addition for 21 min. The resin was washed with methylene chloride 5 times, chloroform 3 times, 10% triethyl amine $(CH_3CH_2)_3N$ in chloroform 3 times and methylene chloride 3 times. The bulk of the resin was stored in methylene chloride. A small aliquot was dried, weighed, and analyzed in the automatic amino acid analyzer. It contained 410 µmoles of valine per gram. That is about one valine per 24 styrene residues. One half of the valine resin was stored in methylene chloride. The remainder of the resin was used for the synthesis of peptides 118-124 and ala$^{114-124}$.

The 118-124 peptide has the sequence valyl-histidyl-phenylalanyl-aspartyl-alanyl-seryl-valine. The following amino acid derivatives were used in the synthesis: Boc-O-benzyl-l-serine, Boc-L-alanine, Boc-β-benzyl-L-aspartic acid, Boc-L-phenylalanine, Boc-N^{im}-DNP-L-histidine. All were synthesized from the corresponding amino acids except the histidine derivative which was obtained commercially. A three-fold molar excess of the serine derivative dissolved in 20 ml of methylene chloride and added to the valine resin. After 7 min, dicyclohexylcarbodimide was added and the coupling allowed to proceed overnight. After two washes with methylene chloride the seryl-valyl resin was treated with trifluoroacetic acid to remove the Boc protecting group and washed in the same manner as the valyl resin. The other amino acid derivatives were added successively in the same manner. Two different procedures were employed for removing the peptide from the resin and deblocking. In the first, an aliquot of 20% of the resin was dried and weighed 1.25 gm. It was suspended in 10 ml of trifluoroacetic acid and HBr was bubbled through the stirred suspension for 90 min. The resin was removed by filtration, washed twice with 10 ml of trifluoroacetic each time. The filtrate and washings were combined and brought to dryness at 30° on a rotary evaporator. These treatments are expected to remove all of the protective groups except the dinitrophenyl group on the histidine.

The peptide was separated from low molecular weight impurities by gel filtration on 1.9 x 150 cm column of Sephadex G10, equilibrated with 0.1 M pyridine acetate, pH 3.2 at 25°. The effluent was analyzed for peptide by the Lowry procedure for dinitrophenyl groups by its absorbance at 390 nm. The major peak was pooled and lyophilized. A small trailing shoulder was discarded.

The dinitrophenyl group was removed by thiolysis with mercaptoethanol. The peptide was dissolved in 10 ml of phosphate buffer 0.1 M, pH 8.0 and a 100-fold mole excess of mercaptoethanol was added. The pH was brought back to 8.0 with 50% NaOH. After the solution stood overnight it was extracted with six 10 ml portions of ethyl acetate. This removed much but not all of the color. The aqueous layer was again placed on the Sephadex G10 column. Most of the yellow didnitophenyl derivative was separated from the peptide.

The material from the second gel filtration was analyzed on the amino acid analyzer and gave the following composition: aspartic acid 1.06, serine 1.01, alanine 1.03, valine 1.89, phenylalanine 0.96 and histidine 0.84. The overall yield was 61% based on the valine resin.

About 1/3 (51 mg) of the peptide was purified further by ion exchange chromatography on a 0.9 x 150 cm column of SP Sephadex

eluted with a gradient of pyridine acetate running between 0.5 M pH 3.6 and 2.0 M, pH 5.9 at 55°. The elution profile was determined by hydrolyzed ninhydrin analysis. A single sharp peak was found and the tubes near the center of the peak were pooled to yield 31 mg of material which has an amino acid composition of aspartic acid 1.06, serine 1.02, alanine 1.04, valine 1.90, phenylalanine 1.00 and histidine 0.97.

A second preparation was made from 1/4 of the remaining peptide 118-124 resin. The dinitrophenyl group was cleaved before the peptide was removed from the resin by adding 10 ml of dimethyl formamide to the 1.13 grams of dried resin. After the resin was wetted the dimethyl formamide was filtered off and 10 ml of 0.4 M thiophenol in dimethyl formamide was added. The release of dinitrophenyl groups was followed spectrophotometrically at 337 nm and indicated that the reaction was complete in 4 min. It was allowed to continue for 30 min and the resin was washed with dimethyl formamide and twice with methylene chloride and dried. The peptide was cleaved as previously and separated on Sephadex G10. The amino acid composition of the material was aspartic acid 1.10, serine, 1.03, alanine 1.04, valine 1.91, phenylalanine 1.01 and histidine 0.91. Yield based on valine resin was 80.5%. One half of the material (99 mg) was chromatographed on a 1 x 145 cm column of SP Sephadex using a gradient of pyridine acetate from 0.25 M, pH 3.50 to 1.00 M, pH 4.50. The fractions were monitored as previously and also by the fluorescamine procedure. The dinitrophenylated impurities eluted before the peptide which had a small trailing shoulder. The main peak was pooled and dried and dissolved in 8.0 ml water adjusted to pH 3.0 with 5.5 ml of glacial acetic acid. The amino acid analysis was aspartic acid 1.12, serine 1.03, alanine 1.02, valine 1.90, phenylalanine 1.03 and histidine 0.90. A yield of 75% was obtained.

Racemization, i.e., formation of D amino acids, can occur during synthesis. Accordingly, each of the final materials was examined enzymatically for its content of D amino cids. Within the error of the method (2-3%) no D amino acids were found.

Peptide ala$^{114-124}$, gly-tyr-val-pro-val-his-phe-asp-ala-ser-val, was prepared by similar means. The material was successfully chromatographed on a 1.0 x 165 cm column of SP Sephadex which had been equilibrated at 50° with 0.05 m pyridine acetate pH 3.00. Elution was at 16 ml/hr with a linear gradient running between 0.05 m pyridine acetate pH 3.00 and 0.25 m pyridine acetate pH 3.75. Its amino acid analysis was aspartic acid 0.99 (Theory, 1.0), serine 0.99 (1.0), proline 0.95 (1.0), alanine 2.06 (2.0), valine 3.15 (3.0) tyrosine 1.00 (1.0), phenylalanine 0.97 (1.0). The tyrosine content by digestion with aminopeptidase M was 1.00. This indicates an absence of racemization and of residual blocking groups remaining on the tyrosine. A spectrophotometric titration also confirmed that the tyrosine was in the proper form.

B. Factors Affecting the Precipitin Reaction

Summary

The precipitin reaction is usually used as a standard for determination of the amount of antibody in a system. In the oxidized ribonuclease immune system the reaction is quite sensitive to the pH and to a lesser extent to the amount of nonspecific protein in the system. The optimal pH for this system is 7.4 $\pm$ 2. Other systems probably have wider optimal pH ranges.

Effect of Antibody Purification

The precipitin reaction is one of the most accurate measures of the reaction between antigen and antibody. It usually serves as the standard for the other methods used for estimating the immune reaction. A difficulty with the procedure became manifest when we found that the precipitin reaction with peptide 105-124 coupled to bovine gamma globulin fails to go into antigen excess. This reaction forms the basis for the determination of the amount of antibody directed to this region. We also found that the precipitin reaction of our immune system was not the same in whole serum as in antiserum which had been fractionated with sodium sulfate. Immune serum gives a precipitin curve of the expected shape which falls off quite promptly in antigen excess. Antibodies which have been prepared from the same serum by sodium sulfate fractionation give a precipitin curve which does not go into inhibition in antigen excess (Fig. 21). Such curves often yield appreciably more "immune" precipitate than is given by a corresponding amount of antiserum. For example, the experiment graphed in Fig. 21 would give 1.0 mg/ml for the precipitable antibody in the undiluted serum and 1.2 mg/ml in the sodium sulfate fraction when calculated back to the corresponding original serum volume.

The original observations were on serum which had been stored frozen for about 1/1/2 yr. A fresh bleeding which had never been frozen behaved in a similar manner (Fig. 22). The immune precipitate is, however, partly soluble in extreme antigen excess. The problem was not associated with bacterial contamination. We could not culture any bacteria from the samples. Material kept in 0.02 sodium azide and experiments performed in azide gave similar precipitin curves. Varying the time (3 to 7 days) the immune reaction was stored before centrifugation did not affect the results. To rule out the possibility that traces of metals were picked up during the fractionation procedure the soidum sulfate fraction was treated with 2 mM ethylenediamine tetraacetate. No changes in the precipitin curve occurred. We did not find the system sensitive to changes in ionic strength between 0.1 and 1 m NaCl. The effect was not changed if the order of addition of antigen and antibody was reversed or if the reactants were mixed more vigorously during the addition of antigen to antibody.

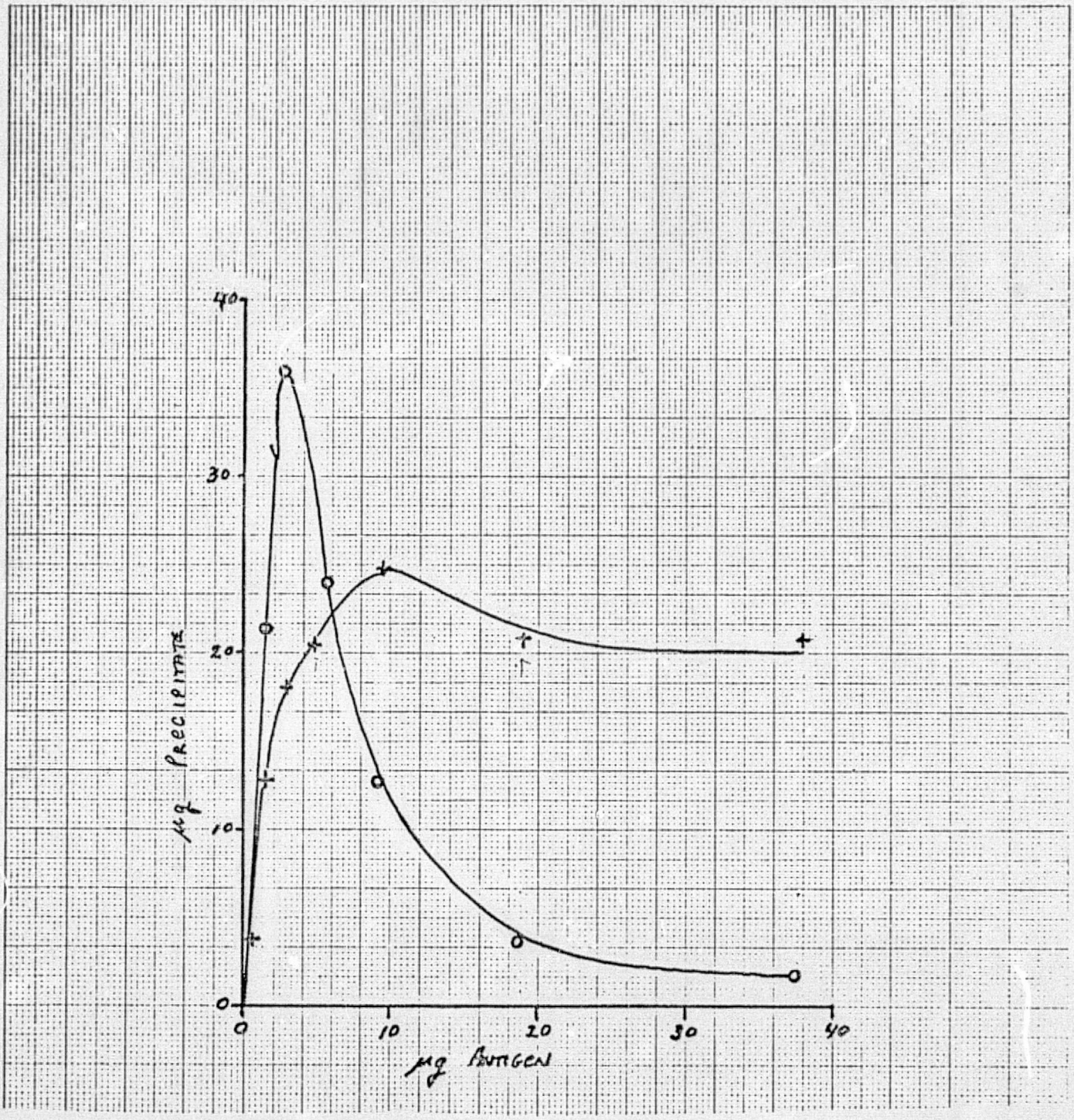


Fig. 21. Precipitin reaction of serum (o) and sodium sulfate fraction (+) from bleeding 23 goat anti-oxidized RNase. Serum diluted 1 to make 3. Gamma globulin fraction diluted 1 to make 8 in relation to original serum volume.

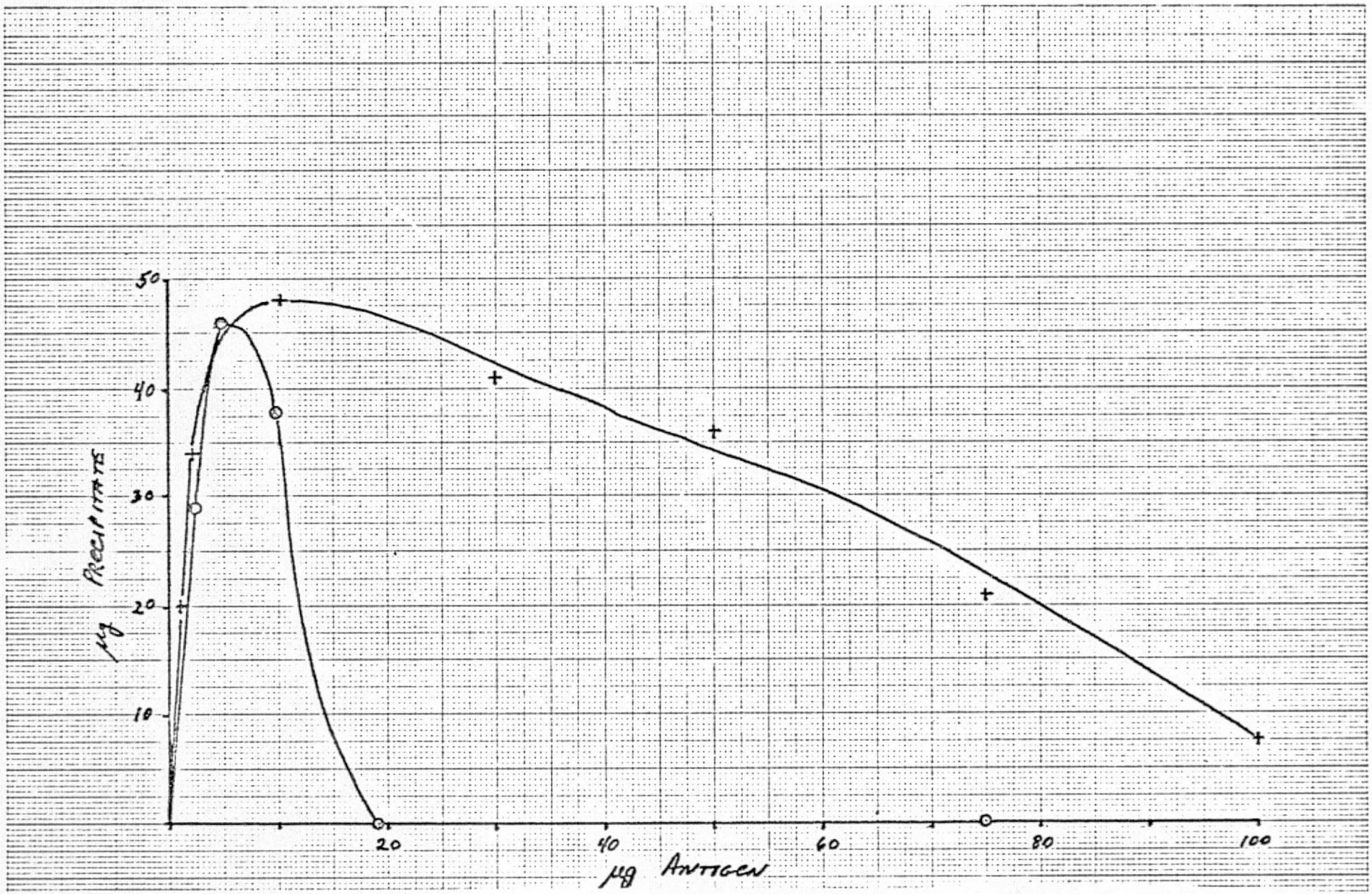


Fig. 22. Precipitin reaction of fresh unfrozen serum (0) and the corresponding sodium sulfate fraction (+) from bleeding 28. Unusually large amounts of antigen were used. The serum was used undiluted and the sodium sufate fraction was diluted 1 to make 2.125.

It seemed that the effect might be due to removal of antibodies or other substances in the supernatant of the sodium sulfate fraction. Accordingly, the dialyzed supernatant from the sodium sulfate fraction was added to a precipitin experiment. It almost completely inhibited precipitation in the dilute systems tried. The supernatant from non-immunized goats had a similar effect (Fig. 23). In a control system, goat anti human serum albumin, the supernatant was without effect. Experiments were also done adding the supernatant from sodium sulfate fractionation of normal serum. With more concentrated solutions of sodium sulfate fraction, addition of supernatant returned the precipitin curve to its normal form (Fig. 24). Since supernatant fractions from normal animals as well as from immune animals altered the reaction, experiments were done to determine whether specific proteins were responsible for the effect. In preliminary experiments, beta amylase, human serum albumin (3X crystallized) and Cohn fraction IV from rabbit serum each restored the precipitin reaction to normal.

We then became concerned that the observations could be due to partial denaturation of the immunoglobulin fraction during preparation and wondered if the precipitation in the antigen excess region was specific. That is, whether the sodium sulfate fraction from oxidized ribonculease antiserum non-specifically added to other immune precipitates. For this an experiment was done using goat antiserum to human serum albumin. Addition of the sodium sulfate precipitate to the reaction at 4 points in antigen excess did not bring down additional precipitate. This makes denaturation a less likely possibility. The sodium sulfate fraction also failed to coprecipitate with the goat anti lysozyme system. A precipitin curve was done with a sodium sulfate fraction which had been ultracentrifuged for 1 hr at 10500 G in the hope that any aggregated, partially denatured protein would be removed. This treatment did not affect the precipitin reaction.

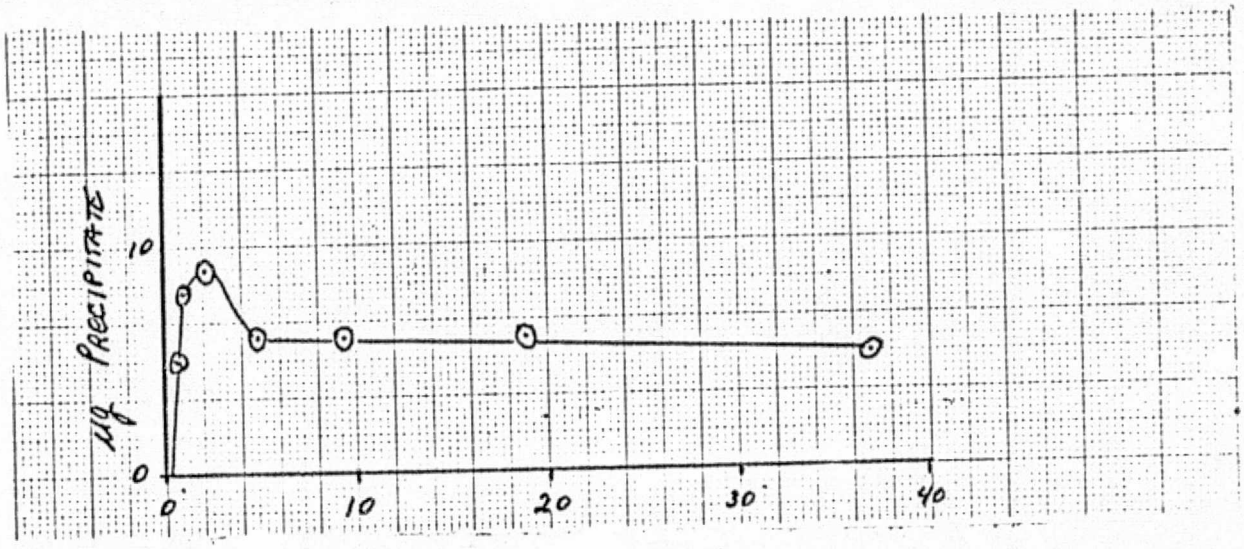


Fig. 23. Effect of normal gamma globulin on the precipitin reaction of antibody precipitated with sodium sulfate from bleeding 23 at a dilution of 1 to make 8 in relation to original serum volume.

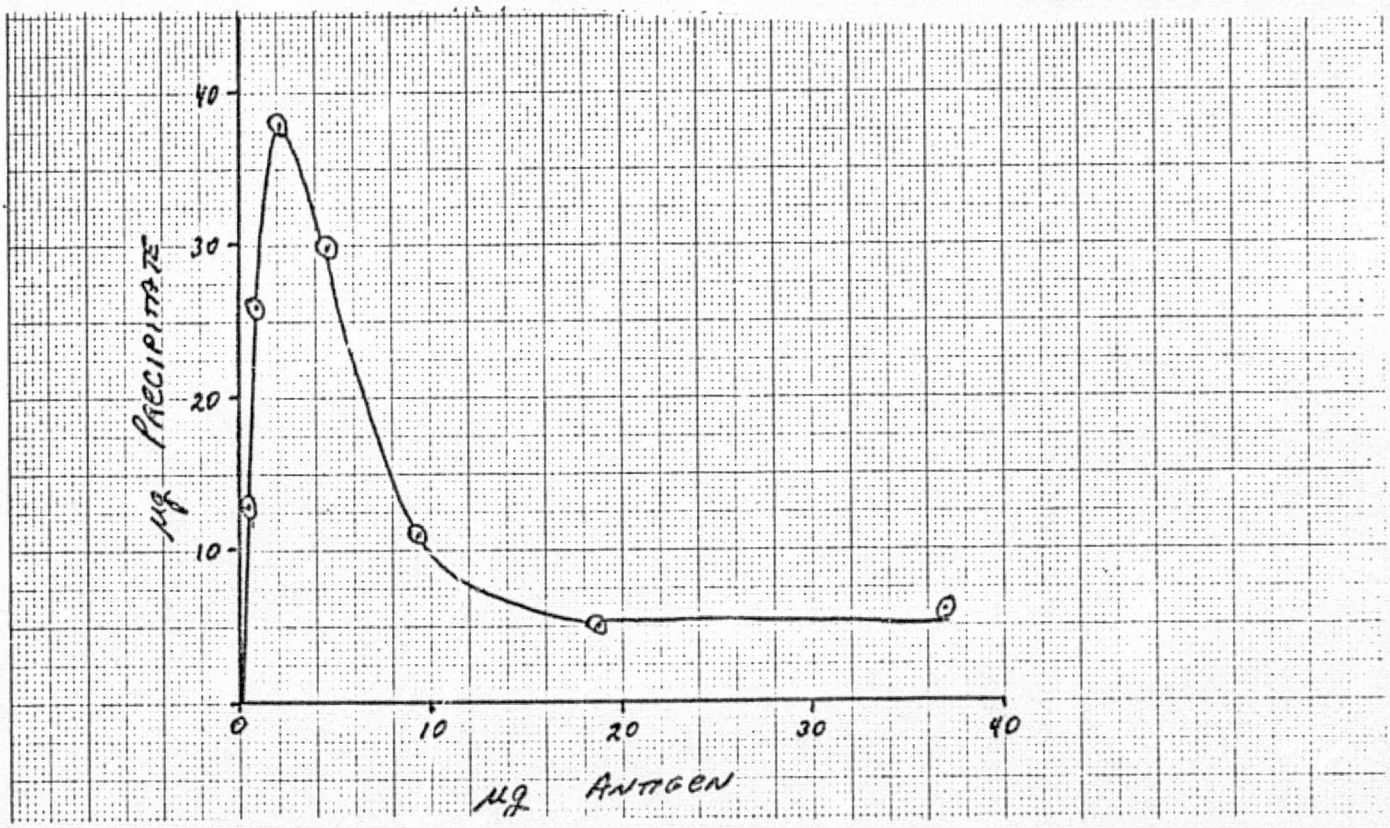


Fig. 24. Precipitin studies with concentrated sodium sulfate fraction and dialyzed supernatant solution. The antibody fraction was diluted one to make two in relation to the original serum.

pH Studies

It is generally found that immune systems have a wide range of pH over which the immune reaction occurs. For example, Nicklin and Stephen (1973) found that precipitin reaction of rabbit antisera to human serum albumin was independent of pH between pH 5 and 9 and for some immunization schedules between pH 4 and 10 (Fig. 25). Antibody to lysozyme had a similar broad zone of pH independence. Preliminary studies suggested that the present system had a great pH dependence. Precipitin analyses were done at two points of the region of antigen excess with the sodium sulfate precipitate and at a point with the antiserum. Three buffer systems were used to study the pH range which extended from 4 to 9. Sodium acetate-acetic acid mixtures were employed at acid pH. Potassium phosphate was used at neutral pH and tris-acetic acid was used at alkaline pH. In all cases the final buffer concentration was 0.05 m in 0.15 m sodium chloride.

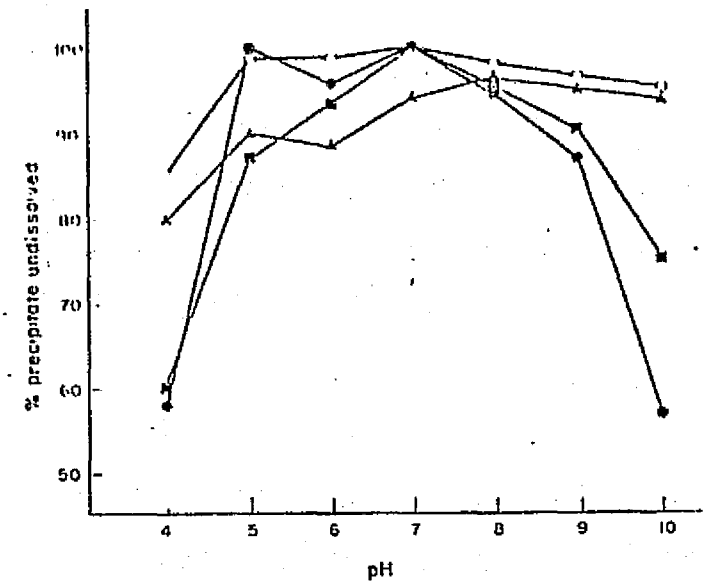


Fig. 25. Comparison of the solubilities of antigen/antibody precipitates derived from CFA-induced (-o-o-o-), schedule I (-o-o-o-), schedule II (-Δ-Δ-Δ-) and schedule III (-▣-▣-▣-) anti-HSA sera. The precipitates were formed at optimum proportions at pH 7.0 and equal amounts exposed to fixed volumes of buffers of constant (0.26) ionic strength and pH values 4-10. Precipitates were washed and residual protein contents measured (from Nicklin and Stephen, Immunochem., 10, 717 (1973)).

In both the whole serum (Fig. 26) and the sodium sulfate fraction (Fig. 27) the precipitin reaction is quite sensitive to the pH. It is clear that the pH must be controlled much more careully than in ordinary precipitin reactions. This is an important consideration for both in-flight and ground based detection systems. The shape of the precipitin curve with the sodium sulfate fraction is not due to pH effect. A precipitin curve where the pH was held at 7.58 $\pm$ .01 had the same shape as the other curves with the sodium sulfate fraction. Since the system is so sensitive to pH an experimend with added protein was performed with careful control of the pH which was held at 7.56 $\pm$ .03. The amount of precipitate recovered is very dependent upon the concentration of albumin used (Fig. 28).

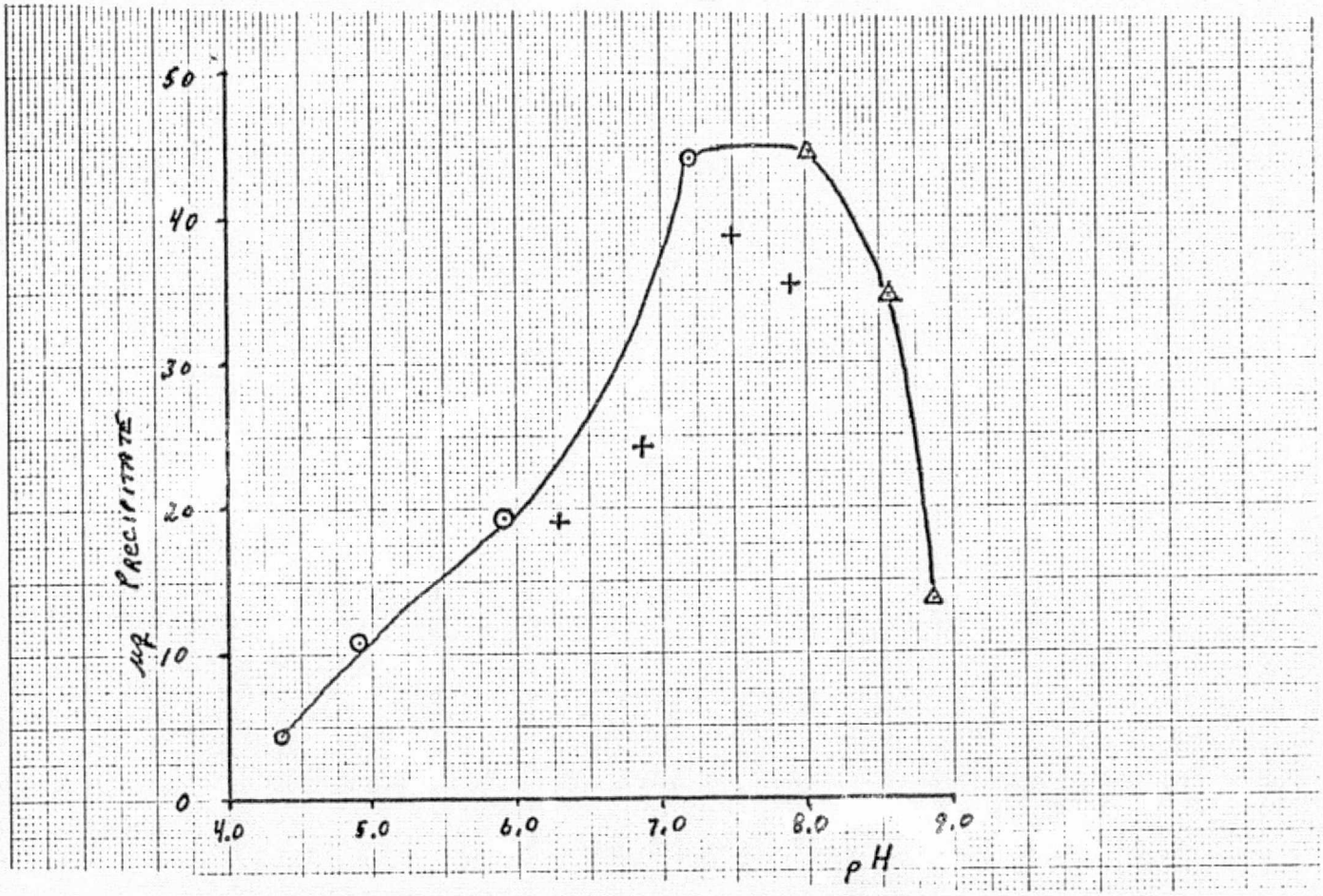


Fig. 26. Dependence of amount of immune precipitate upon pH. Serum from bleeding 23 diluted 1 to make 3 with acetate (o), phosphate (+) or tris (△) buffers. The pH plotted was determined on the supernatant solution from the reaction.

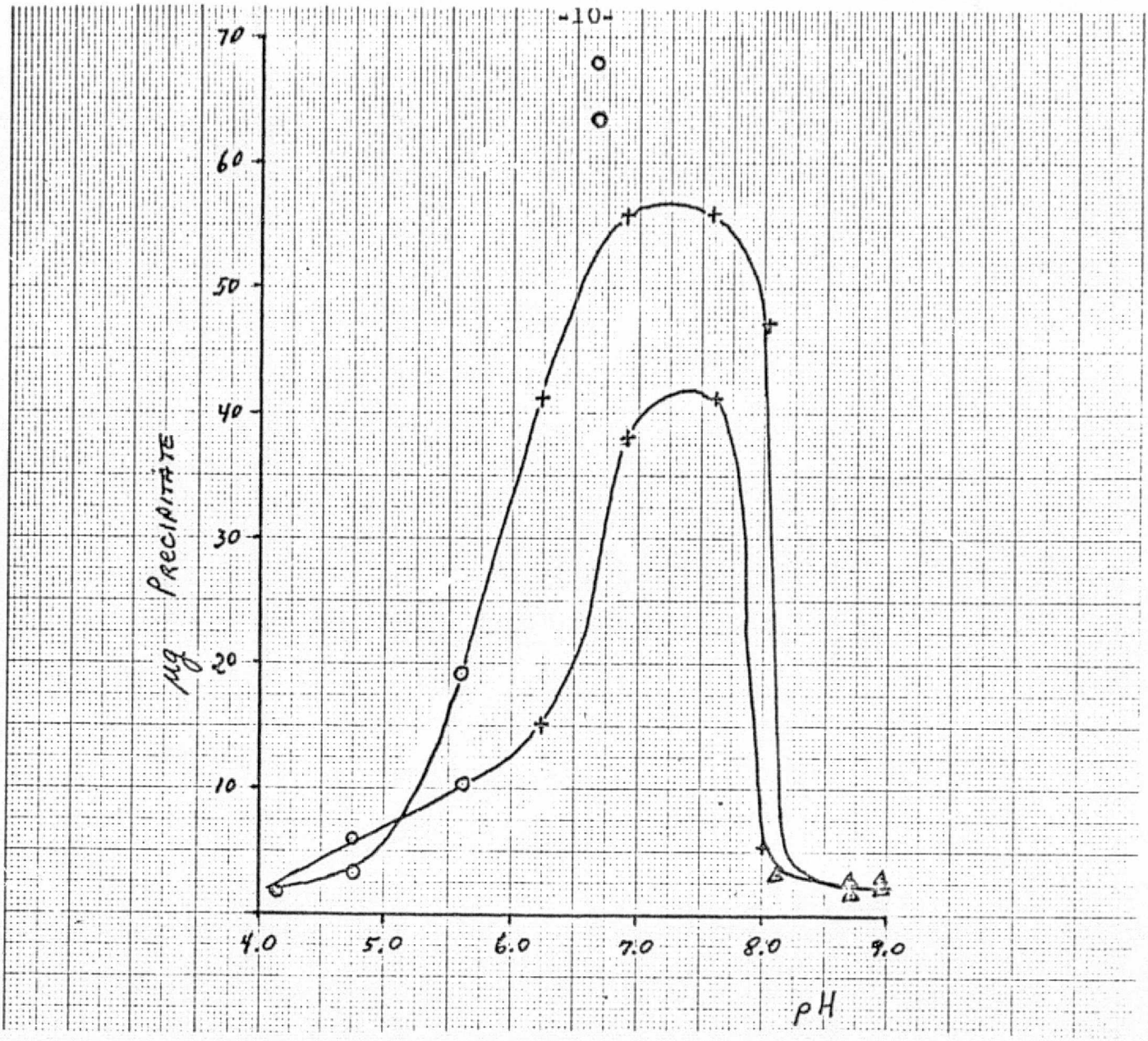


Fig. 27. Effect of pH on the precipitation of antibody from a sodium sulfate fraction of bleeding 23. The upper curve is with 4 µg of antigen per tube and the lower curve is with 18 µg.

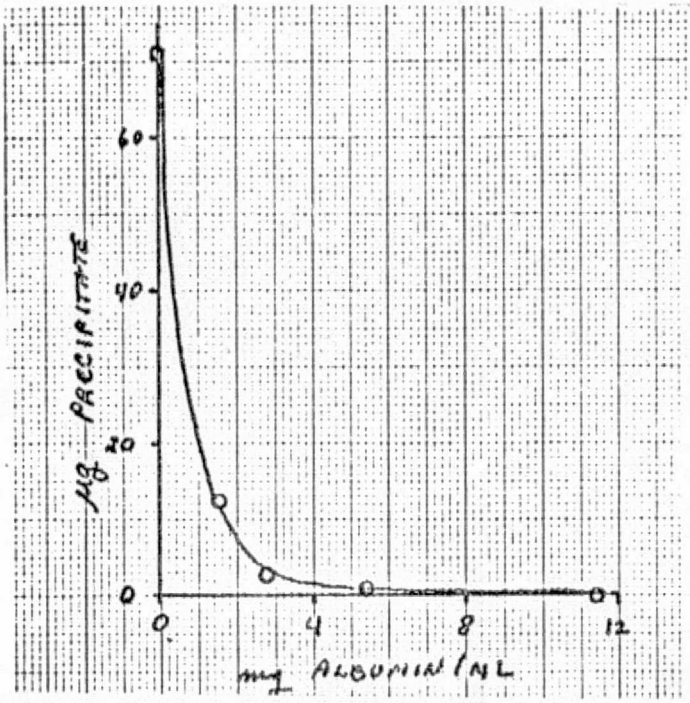


Fig. 28. Effect of albumin concentration on amount of immune precipitate formed. O-RNase (4 g) was mixed with sodium sulfate fractionated antiserum (bleeding 23) diluted 1 to make 3.

The effect seems to have developed during the course of immunization. This effect was not discernable in a bleeding taken at 62 weeks but was quite apparent at 77 weeks. Two other goats immunized with unfolded ribonuclease have immunoglobulin which behaves in a similar manner.

To help rule out artifacts a sample of goat anti lysozyme has been fractionated with sodium sulfate and precipitin reactions done (Fig. 29). It is apparent that the precipitin curve of the sodium sulfate fraction drops more rapidly in antigen excess in the lysozyme system than in the oxidized ribonuclease system. However, it is clear that the shape of the precipitin curve of goat anti lysozyme is altered by sodium sulfate fractionation.

These findings are of significance regarding electrophoretic separation of antibody in space for the following reasons: (1) any evaluation of separated fractions from the present immune system whether ground based or in-flight must be done within a fairly narrow pH range (pH 7.4 $\pm$ .2). This may not hold for other immune systems. These systems contain large numbers of components and it may be that the broad pH range reflects this large number of components. (2) The amount of immune precipitate is influenced by the concentration of non-specific protein in the system.

The pH study gives a more rational basis for our finding that pH's of 4.5 to 5.0 will dissociate antibody from immunoabsorbants (next section). These partially purified antibodies should provide material of less complexity for separation experiments and furnish standard materials for assays.

Other methods such as passive cutaneous anaphylaxis and hemagglutination and their inhibition have been used.

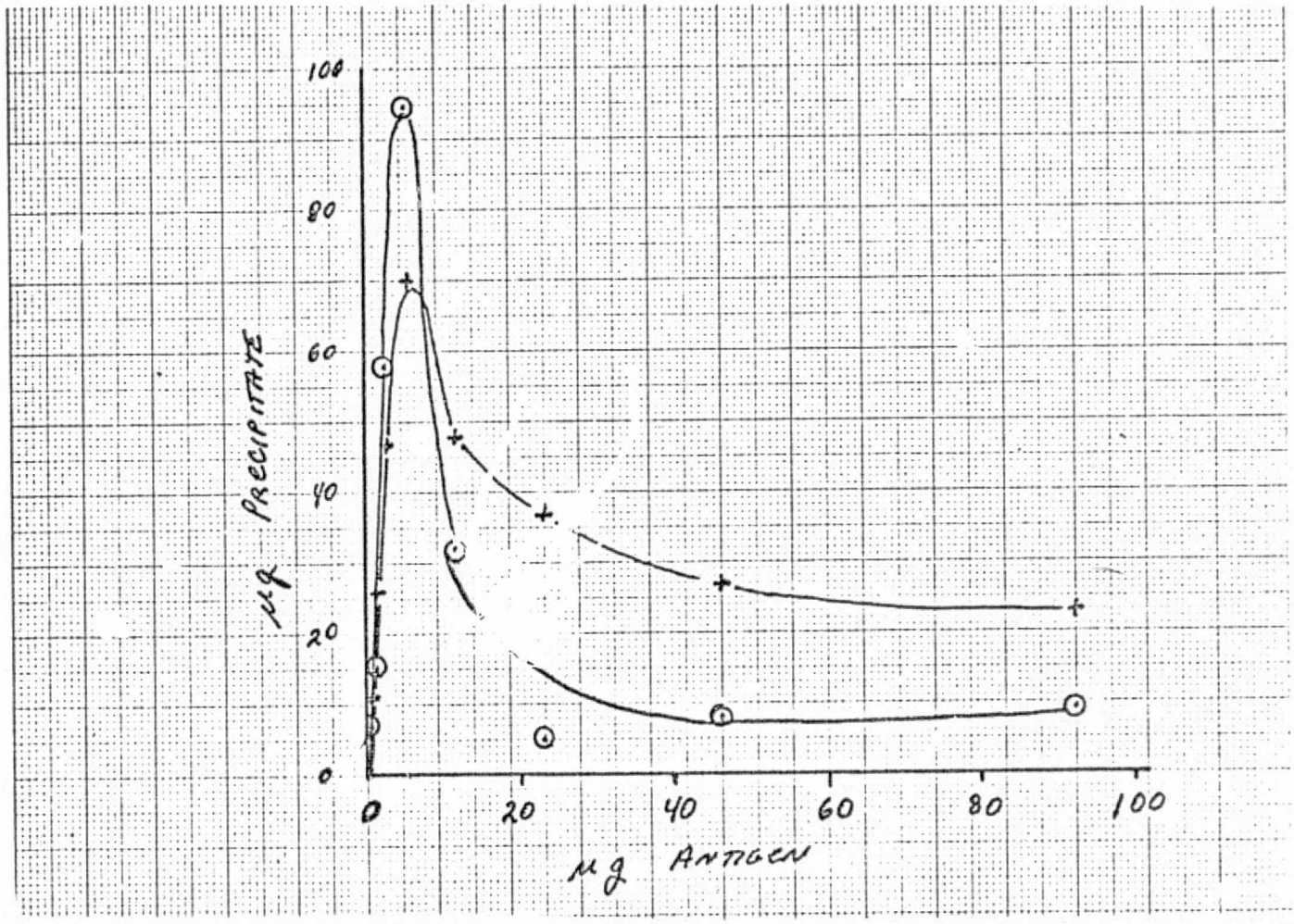


Fig. 29. The precipitin reaction of goat antibody to hen egg white lysozyme before (o) and after (+) fractionation with sodium sulfate.

IV. COMPARISON OF METHODS OF SEPARATING ANTIBODIES

Summary

Conventioanl separation procedures with salts such as sodium or ammonium sulfate are useful in separating antibodies from other plasma proteins but give no separation of antibodies from one another. Chromatographic procedures give some additional fractionation especially between the immunoglobulin classes. The methods are useful for gram amounts of material. Immunoabsorption gives good separation of the antibodies to different areas of the antigen oxidized ribonuclease. The stability of antibodies prepared by immunoabsorption is decreased. Typical separations involve tens of milligrams. Isoelectric focusing in a column stabilized by a sucrose density gradient gives partial separation of antibodies. Antibodies became insoluble under the conditions of the separation and tended to settle in the column. About 20 mg of material were used in the experiment reported. Isoelectric focusing in polyacrylamide gels provides an excellent separation of antibodies on an analytical scale (10-50 μg of material).

Conventional Fractionation

The antibody fraction may be separated in good yield and purity from immune sera by precipitation with salts or by chromatography. Fractionation with sodium sulfate consists of a 3 times repeated precipitation with 1 M Na_2SO_4. To 50 ml of antiserum 7.10 g of solid Na_2SO_4 was added slowly. Initially more salt was not added until the previous addition had dissolved. After all the sodium sulfate was added the suspension was stirred for 1 hr at room temperature and centrifuged at 12,000 x g for 30 min at 20°. The precipitate was dissolved in 50 ml of saline and then reprecipitated. The second precipitate was dissolved in 25 ml saline and the precipitation procedure repeated. The final precipitate was dissolved in 15 ml of phosphate buffered saline and dialyzed.

Fractionation with ammonium sulfate was done by adding 25 ml of saturated ammonium sulfate dropwise to 50 ml of serum. The pH of the resulting suspension was adjusted to 7.8 with 2 N NaOH and the material was stirred for 2 hr at room temperature. After centrifugation the precipitate was brought to its original volume with phosphate buffered saline and reprecipitated. The procedure was repeated twice. The final precipitate was treated as the Na_2SO_4 precipitate. By conventional electrophoresis on cellulose acetate these preparations appear free of contamination with non-γ-globulins. The gamma globulin band while broad and diffuse, does contain 3 faintly visible bands.

We have also made preparations at 0° using half saturated ammonium sulfate at pH 7.1 ± .1. These fractions have a small amount of contamination with other plasma proteins. Material fractionated with sodium sulfate was chromatographically purified for the stability studies. Most of the precipitin studies were done with the sodium sulfate fraction.

Chromatographic Separations

The gamma globulin used for the stability studies was purified further by chromatography on diethylaminoethyl (DEAE) cellulose. Runs were also made with whole antiserum. In a typical experiment 15 ml of goat antiserum was dialyzed against 0.01 M phosphate buffer pH 8.0. After dialysis it was titrated to pH 8.10 and chromatographed on a 58.5 x 3 cm column of DEAE cellulose titrated to this pH. After 308 ml had flowed through the column a linear gradient going from 0.01 to 0.3 M sodium phosphate buffer pH 8.10 was started. It was made with 1 l of each buffer. The first peak (at 260 ml) contained only gamma globulin. Later peaks (at 760 ml and beyond) contained gamma globulin and other plasma proteins. Immunoelectrophoresis indicated that IgG was the only plasma protein in the first peak. This type of material should be adequate for further separation into individual antibodies.

Immunoabsorption (Mikoryak, 1974)

Extensive experiments with antibodies to the two major regions of oxidized ribonuclease, peptides 40-61 and 105-124. A systematic study was made to determine the mildest condition which would yield good recovery of the antibodies with maximum retention of their activity with antigen. Different antibodies were eluted by decreasing the pH from 5.0 to 4.5, by increasing the ionic strength, especially between 0.5 and 1.0 moles and by increasing the temperature from 4° to 22°. Immunoabsorbents eluted at room temperature with 1 M sodium acetate buffer at pH 4.5 yielded little additional protein if a lower pH or 3 M sodium thiocyanate was used. For an immunoabsorbent containing the intact antigen the mildest condition eluting an appreciable portion of antibody was 1 M sodium acetate buffer at pH 4.5 and 4°. About 1/3 of the total precipitable antibody applied to the immunoabsorbent was eluted under these conditions. The precipitability of various preparations ranged from 86 to 94% and was better than that for antibodies eluted at room temperature. Some of the antibodies could be eluded with a buffer whose ionic strength was adjusted with sodium chloride rather than sodium acetate. The concentration of free acetic acid would be low in the buffers adjusted with sodium chloride but quite high in those made with sodium acetate. The antibody fractions obtained by varying the elution conditions differ when examined by polyacrylamide gel isoelectric focusing.

Application of the differential elution conditions to immunoadsorbents containing the antigenic peptide determinants revealed that antibodies were eluted from the peptide 40-61 immunoadsorbent more easily than were the antibodies on the peptide 105-124 immunoadsorbent. The antibodies eluted under similar mild conditions from both an oxidized ribonulcease immunoadsorbent and a peptide 40-61 immunoadsorbent had very similar isoelectric focusing patterns. In addition, antibodies eluted from a peptide 105-124 immunoadsorbent or an oxidized ribonuclease immunoadsorbent under similar, more severe conditions also had very similar isoelectric focusing patterns.

Although the isoelectric points of the two peptides (40-61 and 105-124) are nearly identical, the isoelectric points of antibodies to peptide 40-61 range from 6.6 to 6.9 and the focusing pattern shows restricted heterogeneity (one major and three less prominant bands) while for antibodies to peptide 105-124 the range of isoelectric points is 5.8 to 6.4 and the focusing pattern is more heterogeneous (seven to nine bands). The specificity of the immunoadsorption procedure was examined using the two peptide immunoadsorbents sequentially. The eluted antibodies have the same isoelectric focusing patterns regardless of which order was employed.

Antibody prepared by elution from an immunoadsorbent containing the synthetic peptide ala^{114}114-124, which represents approximatelyone-half of the 105-124 determinant, has an isoelectric focusing pattern which resembles that of antibodies eluted from a 105-124 immunoadsorbent. One interpretation of the data which is consistent with previous observations for the 105-124 determinant is that some of the antibodies are directed toward portions of the determinant which are not contiguous in the sequence.

Isoelectric Focusing in a Liquid System

Twenty mg of antibody to the carboxyterminal region of oxidized ribonuclease was placed in a 110 ml LKB column for liquid isoelectric focusing. The sample was applied mid-column in an initially discontin uous gradient made with 0% and 46% sucrose. The final concentration of pH 3-8 ampholyte was 0.8%. Initial voltage of 200 volts was gradually increased until at the termination of the experiment (48 hr) it was 600 volts. Four major and 2 minor components were partially separated (Fig. 30). Some of the antibody became insoluble during the procedure. The final yield was 10 mg of antibody.

The concentrated, dialyzed fractions were examined by isoelectric focusing in polyacrylamide gel. While a majority of each fraction was the expected component, traces of the other three major antibodies were found in each case.

Gel Isoelectric Focusing

The procedure was performed at 4°. The gel tubes were inserted in the bottom of the upper electrode vessel which contained 0.5% ethylene diamine and extended into the lower electrode vessel which contained 0.5% phosphoric acid. The gels, usually six were run at a time, were prefocused for 30-60 min at 60 V and ar initial current of 5 ma using a power supply which provided 50 pulses per second (Ortec 4100 Pulsed constant power supply).

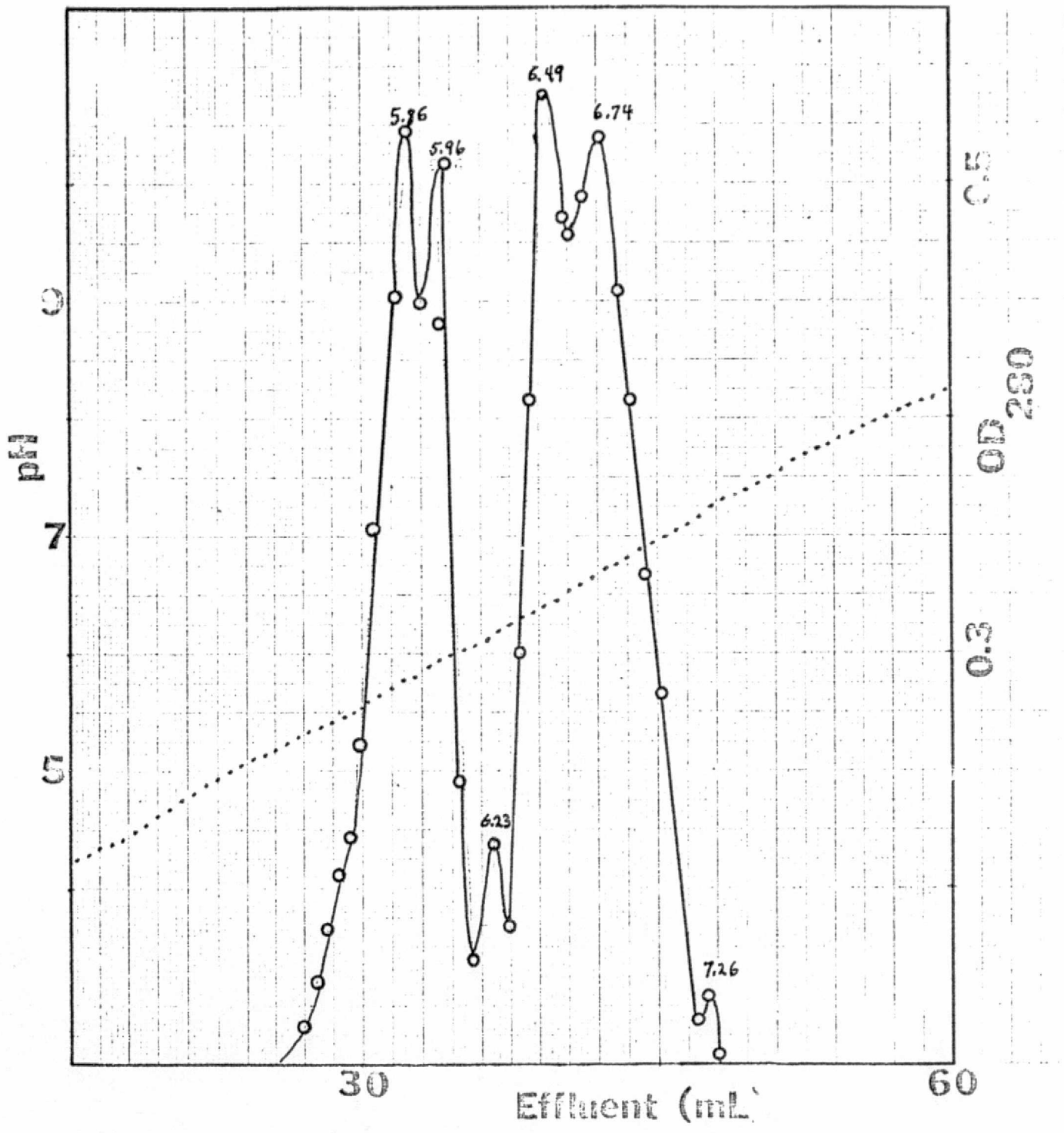


Fig. 30. Liquid Isoelectric focusing of antibodies to the carboxy terminus of oxidized ribonuclease

The samples contained between 25 and 100 µg of protein in 50 µl of solution which which was added 5 µl of saturated sucrose containing meta cresol purple dye. The dense sample solutions were carefully layered on top of the gels. The focusing was started at 60 V and the voltage was gradually increased in 20 V increments every 10-15 min until 150 V was reached. Focusing proceeded for 20-22 hr at which time the dye was an orange-pink band near the bottom of the gel.

The gels were removed from the tubes and stained immediately with 0.1% Coomassie blue in acetic acid:ethanol:water (4:36:40) for one hr. The initial destaining was accomplished in a solution containing the same proportions of solvents but without the stain. Then the alcohol content was gradually decreased from 45 to 25%. The gels were stored in 5% acetic acid.

Two major areas of oxidized ribonuclease are antigenic, peptides 40-61 and 105-124. The former has 2 to 4 major components all of which lie alkaline to the 6-8 major components of the 105-124 region (Fig. 31). By selection of elution conditions one can elute antibodies which appear similar to those to the 40-61 region from an immunoadsorbent prepared from oxidized ribonuclease (Fig. 32). Much of our current interest lies in altering the separation conditions for isoelectric focusing so that better separations can be achieved between the components. This should allow separation of the larger amounts required for preparative work.

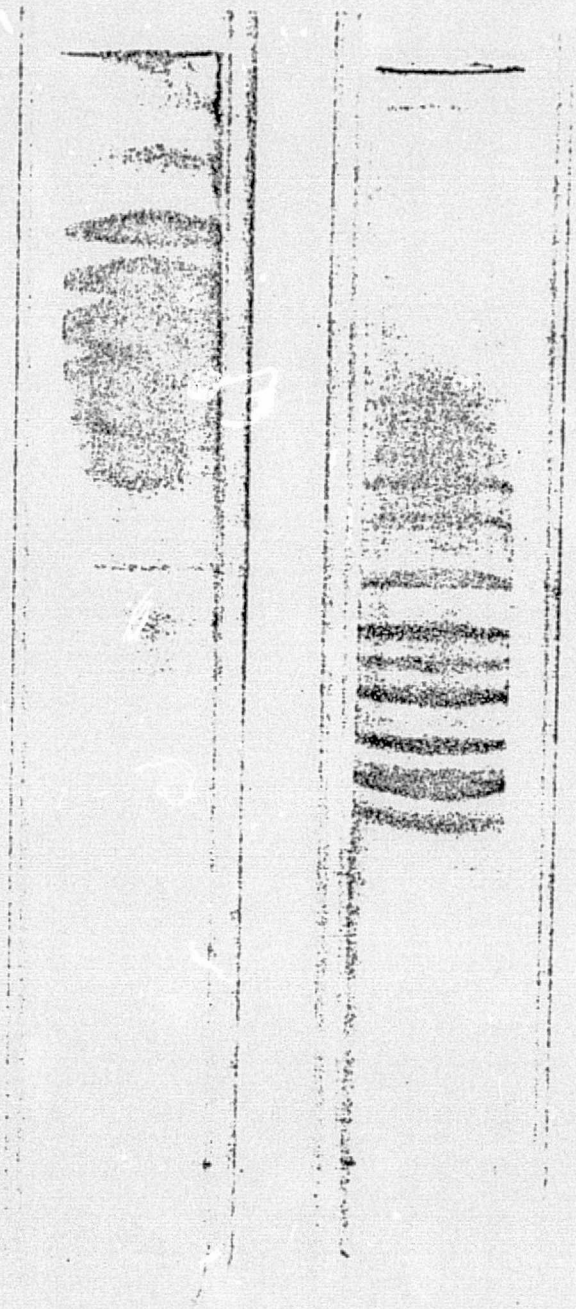


Fig. 31. Isoelectric focusing with the antibodies eluted from the immunoadsorbents containing the antigenic determinants, peptide 40-61 or peptide 105-124. Focusing in polyacrylamide gel was performed in the pH range 5-8 (bottom to top of gel). On eht left is the gel obtained with the antibodies from the peptide 40-61 immunoadsorbent eluted at 4° with 1 M NaOAc, pH 4.5 (70 µg); on the right is the gel obtained with the antibodies from the peptide 105-124 immunoadsorbents eluted at room temperature with 1 M NaOAc, pH 4.5 (30 µg).

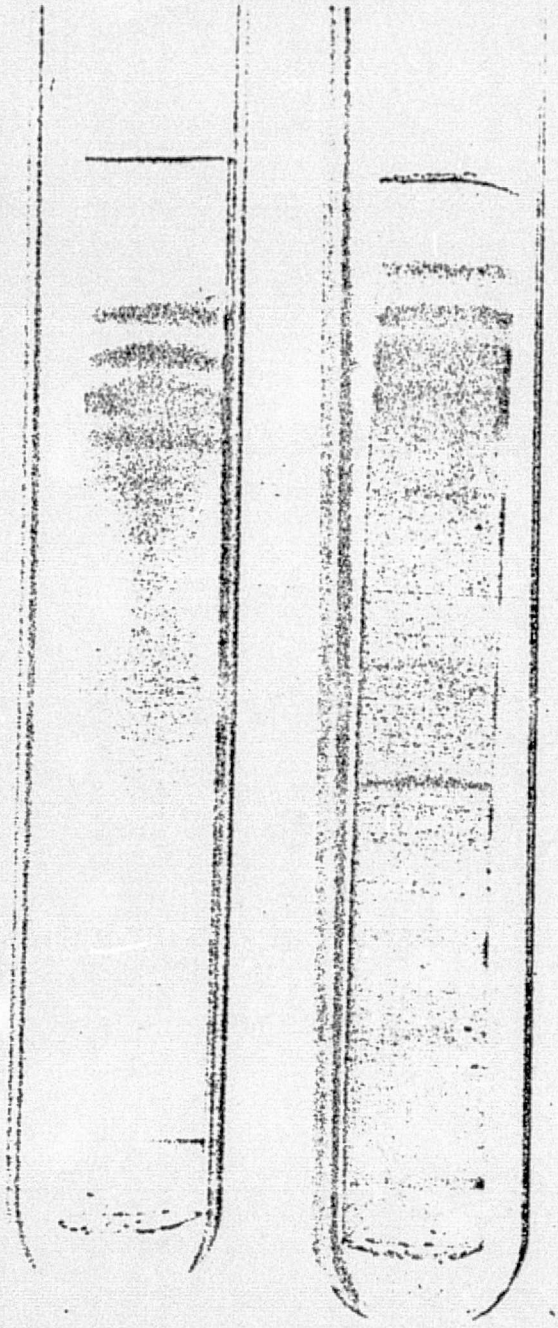


Fig. 32. Comparison of antibodies eluted from the 40-61 immunoadsorbent and an antibody fraction eluted differentially from the oxidized ribonuclease immunoadsorbent. Polyacrylamide gel isoelectric focusing was performed in the pH range 5-8 (bottom to top of gel). On the left is the gel obtained with the antibody fraction eluted from the peptide 40-61 immunoadsorbent at 4° with 1 M NaOAc, pH 4.5 (70 µg) and on the right is the gel obtained with the antibody fraction eluted from the oxidized ribonuclease immunoadsorbent at 4° wtih 0.15 M NaCl, 0.1 M NaOAc, pH 5.0 (70 µg).

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